

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 97/15692

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Inter. nal Application No  
PCT/US 97/15692

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SAMULSKI R J ET AL: "HELPER-FREE STOCKS OF RECOMBINANT ADENO-ASSOCIATED VIRUSES NORMAL INTEGRATION DOES NOT REQUIRE VIRAL GENE EXPRESSION" JOURNAL OF VIROLOGY, vol. 63, no. 9, 1 September 1989, pages 3822-3828, XP000283159 see page 3822 see abstract ---	
P,X	WO 97 26337 A (AVIGEN INC ;UNIV JOHNS HOPKINS (US)) 24 July 1997 see page 6, line 9 - page 9, line 5 see page 20, line 2 - page 34, line 5 ---	1-10
P,X	HERZOG ET AL: "STABLE GENE TRANSFER AND EXPRESSION OF HUMAN BLOOD COAGULATION FACTOR IX AFTER INTRAMUSCULAR INJECTION OF RECOMBINANT ADENO-ASSOCIATED VIRUS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES,USA, vol. 94, May 1997, pages 5804-5809, XP002054071 see the whole document ---	1-10
P,X	FISHER ET AL: "RECOMBINANT ADENO-ASSOCIATED VIRUS FOR MUSCLE DIRECTED GENE THERAPY" NATURE MEDICINE, vol. 3, no. 3, March 1997, pages 306-312, XP002054072 see the whole document ---	1-10
P,X	KESSLER ET AL: "GENE DELIVERY TO SKELETAL MUSCLE RESULTS IN SUSTAINED EXPRESSION AND SYSTEMIC DELIVERY OF A THERAPEUTIC PROTEIN" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES,USA, vol. 93, November 1996, pages 14082-14087, XP002054073 see the whole document ---	1-10
P,X	WO 97 12050 A (UNIV ROCKEFELLER ;KAPLITT MARTIN J (US); DIETRICH EDWARD B (US)) 3 April 1997 see page 5, line 29 - page 9, line 21 see page 26; example 1 -----	1-10

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/15692

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 C12N15/86 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 34670 A (CHILDRENS HOSPITAL INC) 21 December 1995 see page 5, line 16 - page 9, line 12 see especially page 9, first line see examples 4-7	1-10
X	COOVERT D D ET AL: "GENE THERAPY FOR MUSCLE DISEASES" CURRENT OPINION IN NEUROLOGY, vol. 7, no. 5, October 1994, pages 463-470, XP000673519 see the whole document	1-10
A	US 5 436 146 A (SHENK THOMAS E ET AL) 25 July 1995 see page 5, line 55 - page 6, line 13 see column 9, line 47 - line 62	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

2

Date of the actual completion of the international search

2 February 1998

Date of mailing of the international search report

04.03.98

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
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Authorized officer

Sitch, W

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
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DE	Germany	LK	Sri Lanka	SD	Sudan		
DK	Denmark	LR	Liberia	SE	Sweden		
EE	Estonia			SG	Singapore		



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : C12N 15/86, A61K 48/00		A3	(11) International Publication Number: WO 98/09657
			(43) International Publication Date: 12 March 1998 (12.03.98)
(21) International Application Number: PCT/US97/15692		(74) Agents: KODROFF, Cathy, A. et al.; Howson & Howson, Spring House Corporate Center, P.O. Box 457, Spring House, PA 19477 (US).	
(22) International Filing Date: 4 September 1997 (04.09.97)			
(30) Priority Data:		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
08/708,188 6 September 1996 (06.09.96) US 08/729,061 10 October 1996 (10.10.96) US			
(60) Parent Applications or Grants			
(63) Related by Continuation			
US 08/708,188 (CIP)			
Filed on 6 September 1996 (06.09.96)			
US 08/729,061 (CIP)			
Filed on 10 October 1996 (10.10.96)			
(71) Applicant (for all designated States except US): TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; Suite 300, 3700 Market Street, Philadelphia, PA 19104-3147 (US).		Published	
(72) Inventors; and		With international search report.	
(75) Inventors/Applicants (for US only): WILSON, James, M. [US/US]; 1350 N. Avignon Drive, Gladwyne, PA 19035 (US). FISHER, Krishna, J. [US/US]; 2338 Nashville Avenue, New Orleans, LA 70115 (US).		Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	
		(88) Date of publication of the international search report: 23 April 1998 (23.04.98)	

(54) Title: METHOD FOR RECOMBINANT ADENO-ASSOCIATED VIRUS-DIRECTED GENE THERAPY

## (57) Abstract

A method of prolonging gene expression by reducing immune response to a recombinant adeno-associated virus (AAV) bearing a desired gene administered into the muscle of a mammal is described.

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

Remark : Although claims 7-10 partially, in so far as they relate to in vivo methods, are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern 1al Application No

PCT/US 97/15692

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9534670 A	21-12-95	US 5658785 A	19-08-97
		AU 3124395 A	05-01-96
		CA 2192215 A	21-12-95
		EP 0764213 A	26-03-97
US 5436146 A	25-07-95	NONE	
WO 9726337 A	24-07-97	WO 9726336 A	24-07-97
WO 9712050 A	03-04-97	AU 7246796 A	17-04-97

## DEMAND

Demand under Article 31 of the Patent Cooperation Treaty:  
 • The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty.

For International Preliminary Examining Authority use only	
Identification of IPEA	Date of receipt of DEMAND
<b>Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION</b> Applicant's or agent's file reference GNVPN.019CIP2PCT	
International application No. PCT/US97/15692	International filing date (day/month/year) 04 September 1997 (04.09.97)
(Earliest) Priority date (day/month/year) 06 September 1996 (06.09.96)	
Title of invention Method for Recombinant Adeno-Associated Virus-Directed Gene Therapy	
<b>Box No. II APPLICANT(S)</b>	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.) Trustees of the University of Pennsylvania 3700 Market Street Suite 300 Philadelphia, Pennsylvania 19104-3147 United States of America	
Telephone No.: 215-573-4500	
Facsimile No.: 215-898-9519	
Teleprinter No.:	
State (i.e. country) of nationality: United States of America	State (i.e. country) of residence: United States of America
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.) Wilson, James M. 1350 N. Avignon Drive Gladwyne, Pennsylvania 19035 United States of America	
State (i.e. country) of nationality: United States of America	State (i.e. country) of residence: United States of America
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.) Fisher, Krishna J. 2338 Nashville Avenue New Orleans, Louisiana 70115 United States of America	
State (i.e. country) of nationality: United States of America	State (i.e. country) of residence: United States of America
<input type="checkbox"/> Further applicants are indicated on a continuation sheet.	



**Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE**

The following person is ☒ agent ☐ common representative

and ☒ has been appointed earlier and represents the applicant(s) also for international preliminary examination.

☐ is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

☐ is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

Kodroff, Cathy A.  
Howson and Howson  
Spring House Corporate Center  
P.O. Box 457  
Spring House, Pennsylvania 19477  
United States of America

Telephone No.:  
215-540-9200

Facsimile No.:  
215-540-5818

Teleprinter No.:

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

**Box No. IV STATEMENT CONCERNING AMENDMENTS**

The applicant wishes the International Preliminary Examining Authority\*

(i) ☒ to start the international preliminary examination on the basis of the international application as originally filed.

(ii) ☐ to take into account the amendments under Article 34 of

☐ the description (amendments attached)

☐ the claims (amendments attached)

☐ the drawings (amendments attached)

(iii) ☐ to take into account any amendments of the claims under Article 19 filed with the International Bureau (a copy is attached).

(iv) ☐ to disregard any amendments of the claims made under Article 19 and to consider them as reversed.

(v) ☐ to postpone the start of the international preliminary examination until the expiration of 20 months from the priority date unless that Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

\* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

**Box No. V ELECTION OF STATES**

The following designated States are hereby elected:

(i) ☒ all eligible States (i.e., all designated States bound by Chapter II of the PCT).

(ii) ☐ the States indicated in the Supplemental Box No. V.

## Box No. VI CHECK LIST

The demand is accompanied by the following documents for the purposes of international preliminary examination:

- |  |   |        |
|--|---|--------|
| 1. amendments under Article 34                     |   |        |
| description  | : | sheets |
| claims   | : | sheets |
| drawings   | : | sheets |
| 2. letter accompanying amendments under Article 34 | : | sheets |
| 3. copy of amendments under Article 19             | : | sheets |
| 4. copy of statement under Article 19              | : | sheets |
| 5. other (specify):                                | : | sheets |

For International Preliminary Examining Authority use only

received                      not received

<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
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<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>

The demand is also accompanied by the item(s) marked below:

- |  |  |
|--|--|
| 1. <input type="checkbox"/> separate signed power of attorney      | 4. <input checked="" type="checkbox"/> fee calculation sheet |
| 2. <input type="checkbox"/> copy of general power of attorney      | 5. <input type="checkbox"/> other (specify):                 |
| 3. <input type="checkbox"/> statement explaining lack of signature |  |

## Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

By:

Cathy A. Kodroff  
Cathy A. Kodroff  
Attorney for Applicants

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:

2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):

3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date.

☐ The applicant has been informed accordingly.

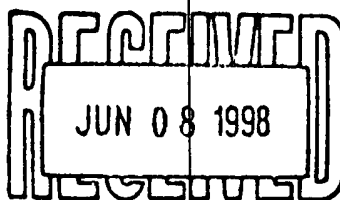
For International Bureau use only

Demand received from IPEA on:

From the  
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

KODROFF, Cathy A.  
Howson and Howson  
Spring House Corporate Cntr.,  
P.O. Box 457  
Spring House, PA 19477  
ETATS-UNIS D'AMERIQUE



PCT

WRITTEN OPINION

(PCT Rule 66)

Date of mailing  
(day/month/year)

03.06.98

Applicant's or agent's file reference

GNVPN.019CIP2PCT

REPLY DUE

within 3 month(s)  
from the above date of mailing

International application no.

PCT/US97/15692

International filing date (day/month/year)

04/09/1997

Priority date (day/month/year)

06/09/1996

International Patent Classification (IPC) or both national classification and IPC

C12N15/86

Applicant

TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA et al.

ENTERED  
DUE 9-3-98

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This report contains indications relating to the following items:

- I ☒ Basis of the opinion
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☒ Lack of unity of invention
- V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☒ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

**When?** See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

**How?** By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

**Also:** For an additional opportunity to submit amendments, see Rule 66.4.  
For the examiner's obligation to consider amendments and / or arguments, see Rule 66.4bis.  
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 06/01/1999

Name and mailing address of the international preliminary examining authority

European Patent Office  
D-80298 Munich  
Tel: (49-89) 2399-0, Tx: 523656 epnu d

Authorized officer / Examiner

SCHEFFZYK, I

Formalities officer (incl. extension of time limits)

Heisel, M



**I. Basis of the opinion**

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*):

**Description, pages:**

1-53 as originally filed

**Claims, No.:**

1-10 as originally filed

**Drawings, sheets:**

1/16-16/16 as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

**IV. Lack of unity of invention**

1. In response to the invitation (Form PCT/IPEA/405) to restrict or pay additional fees, the applicant has:

- ☐ restricted the claims.
- ☐ paid additional fees.
- ☐ paid additional fees under protest.
- ☐ neither restricted nor paid additional fees.

2. This Authority found that the requirement of unity of invention is not complied with for the following reasons and chose, according to Rule 68.1. not to invite the applicant to restrict or pay additional fees:

## WRITTEN OPINION

International application No. PCT/US97/15692

**see separate sheet**

3. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this opinion:

- ☒ all parts.
- ☐ the parts relating to claims Nos. .

### V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

#### 1. Statement

Novelty (N)	Claims	7
Inventive step (IS)	Claims	7-10
Industrial applicability (IA)	Claims	7-10: see item VIII/2).

#### 2. Citations and explanations

**see separate sheet**

### VI. Certain documents cited

#### 1. Certain published documents (Rule 70.10)

and / or

#### 2. Non-written disclosures (Rule 70.9)

**see separate sheet**

### VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

**see separate sheet**

SECTION IV -----

The common inventive concept underlying the present claims can be seen in the use of recombinant adeno-associated virus (rAAV) which is free of contamination with a helper virus for therapeutic purposes. However, the usefulness of such a virus for therapeutic gene delivery to cells, inclusive skeletal muscle cells is already taught in the prior art (see e.g. Coover D.D. et al., Current Opinion in Neurology, vol. 7, no. 5, October 1994, pp. 463-470 (1)).

Thus, the common concept underlying the present claims no longer exists.

Correspondingly, the IPEA is of the opinion that present claims do not relate to one but to two different inventions which are not linked by a common inventive concept as required by Rule 13.1-13.3 PCT, namely:

invention 1: use of rAAV for reducing the immune response to the rAAV when administered into skeletal muscle cell (claims 1, 7-10 and claims 3-6, partly) and

invention 2: use of rAAV for prolonging expression of the transgene when administered into skeletal muscle cell (claim 2 and claims 3-6, partly).

SECTION V -----

Novelty:

The subject-matter of claim 7 is anticipated by the teaching of Coover D.D. et al., Current Opinion in Neurology, vol. 7, no. 5, October 1994, pp. 463-470 (1) wherein the use of rAAV to deliver and express the dystrophin gene in skeletal muscle cells is described. Although (1) is silent with regard to the absence of a cytotoxic immune response the disclosure of (1) is considered to be novelty destroying for claim 7 since all steps defined in the said claim are disclosed in (1). Thus, carrying out the steps defined in claim 7 the expression of the dystrophin gene must inherently take place in the absence of a cytotoxic immune response. Otherwise, claim 7 would be open to an objection under Art. 6 PCT, since at least one feature essential to the performance of the method according to this claim would be missing in claim 7.

Therefore, claim 7 does not comply with the requirements of Art. 33(2)(3) PCT.

Inventive step:

Considering that the use of helper-free stocks of rAAV to deliver therapeutic genes into cells in general as well as into skeletal muscle cells is already taught in the prior art (see e.g. (1), WO 95/34670 (2) and US-A-5 436 146 (3)) the subject-matter of claims 8-10 cannot be considered to be inventive but merely as obvious embodiments to a person skilled in the art which arise out of the knowledge of the prior art (Art. 33(3) PCT).

SECTION VI -----

WO 97/26337	24.07.97	17.01.97	18.01.96
		16.01.97	

Herzog et al. PNAS, vol. 94, May 1997, pp. 5804-5809

Fisher et al., Nature Medicine, vol. 3, no. 3, March 1997,  
pp. 306-312

Kessler et al. PNAS, vol. 93, November 1996, pp. 14082-14087

WO 97/12050	03.04.97	26.09.96	27.09.95
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SECTION VIII -----

- 1). In the absence of a reference point the terms "reducing" and "prolonging" used in claims 1 and 2, respectively, are vague and thus, render the scope of said claims unclear (Art. 6 PCT).

- 2). For the assessment of the present claims 7-10 on the question whether they are industrially applicable, no unified criteria exist in the PCT. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.





✉ EPA/EPO/OEB  
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TX 523 656 epmu d  
FAX (089) 2399-4465

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Patentamt

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Direction Générale 2

## Correspondence with the EPO on PCT Chapter II demands

In order to ensure that your PCT Chapter II demand is dealt with as promptly as possible you are requested to use the enclosed self-adhesive labels with any correspondence relating to the demand sent to the Munich Office.

One of these labels should be affixed to a prominent place in the upper part of the letter or form etc. which you are filing.



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Patentamt

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Patent Office

Office européen  
des brevets

Generaldirektion 2

Directorate General 2

Direction générale 2

### **Schriftverkehr mit dem EPA bei PCT Kapitel II Anträgen**

Um sicherzustellen, daß Ihr PCT Kapitel II Antrag so rasch wie möglich behandelt werden kann, werden Sie gebeten die beige-fügten Klebeschilder bei allen an das EPA München gerichteten Schriftstücken zu verwenden.

Eines dieser Klebeschilder sollte an gut erkennbarer Stelle am oberen Rand der Titelseite des jeweiligen Schreibens angebracht werden.

### **Correspondence with the EPO on PCT Chapter II demands**

In order to ensure that your PCT Chapter II demand is dealt with as promptly as possible you are requested to use the enclosed self-adhesive labels with any correspondence relating to the demand sent to the Munich Office.

One of these labels should be affixed to a prominent place in the upper part of the letter or form etc. which you are filing.

### **Correspondance avec l'OEB en ce qui concerne les demandes PCT, chapitre II**

Pour être sûr que votre demande PCT, chapitre II, sera traitée aussi vite que possible, vous êtes priés d'utiliser les étiquettes autocollantes ci-jointes avec le courrier relatif à la demande envoyé à l'Office à Munich.

Une de ces étiquettes devrait être apposée à un endroit bien visible, à la partie supérieure de l'en-tête de la lettre ou du formulaire etc. que vous déposez.

From the  
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

to:

KODROFF, Cathy A.  
Howson and Howson  
Spring House Corporate Cntr.,  
P.O. Box 457  
Spring House, PA 19477  
ETATS-UNIS D'AMERIQUE

DEC 21 1998

PCT

NOTIFICATION OF TRANSMITTAL OF  
THE INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing  
(day/month/year)

15.12.98

Applicant's or agent's file reference  
GNVPN.019CIP2PCT

IMPORTANT NOTIFICATION

International application No.  
PCT/US97/15692

International filing date (day/month/year)  
04/09/1997

Priority date (day/month/year)  
06/09/1996

Applicant

TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/

European Patent Office  
D-80298 Munich



Authorized officer

Houyez-Stevens, M



# PATENT COOPERATION TREATY

## PCT

### INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference GNVPN.019CIP2PCT	<b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (PCT/IPEA/416)	
International application No. PCT/US97/15692	International filing date (day/month/year) 04/09/1997	Priority date (day/month/year) 06/09/1996
International Patent Classification (IPC) or national classification and IPC C12N15/86		
Applicant TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

- ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☒ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☒ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand 02/04/1998	Date of completion of this report 15.12.98
Name and mailing address of the IPEA/  European Patent Office D-80298 Munich Tel: (+49 89) 2399-0, Tx: 523656 ppmu d	Authorized officer  SCHEFFZYK, I



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/US97/15692

**I. Basis of the report**

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.)*:

**Description, pages:**

1-53 as originally filed

**Claims, No.:**

1-10 as originally filed

**Drawings, sheets:**

1/16-16/16 as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:  
☐ the claims, Nos.:  
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

**IV. Lack of unity of invention**

1. In response to the invitation to restrict or pay additional fees the applicant has:

- ☐ restricted the claims.  
☐ paid additional fees.  
☐ paid additional fees under protest.  
☐ neither restricted nor paid additional fees.

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/US97/15692

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.
3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:

**see separate sheet**

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos. .

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

**1. Statement**

Novelty (N)	Yes:	Claims 1-6, 8-10
	No:	Claims 7
Inventive step (IS)	Yes:	Claims 1-6
	No:	Claims 7-10
Industrial applicability (IA)	Yes:	Claims 1-6
	No:	Claims 7-10: see section VIII/2),

**2. Citations and explanations**

**see separate sheet**

**VI. Certain documents cited**

**1. Certain published documents (Rule 70.10)**

and / or

**2. Non-written disclosures (Rule 70.9)**

**see separate sheet**

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/US97/15692

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**VIII. Certain observations on the international application**

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

**see separate sheet**

SECTION IV-----

The common inventive concept underlying the present claims can be seen in the use of adeno-associated virus (rAAV) which is free of contamination with a helper virus for therapeutic purposes. However, the usefulness of such a virus for therapeutic gene delivery to cells, such as for instance skeletal muscle cells, is already taught in the prior art. (see e.g. Coover D.D. et al., Current Opinion in Neurology, vol. 7, no. 5, October 1994, pp. 463-470 (1)).

Thus, the common inventive concept underlying the present claims no longer exists.

Correspondingly, the IPEA is of the opinion that present claims do not relate to one but to two different inventions which are not linked by a common inventive concept as required by Rule 13.1-13.3 PCT, namely:

invention 1: use of rAAV for reducing the immune response to the rAAV when administered into skeletal muscle cells (claims 1, 7-10, complete and claims 3-6, partly) and

invention 2: use of rAAV for prolonging expression of the transgene when administered into skeletal muscle cells (claim 2 complete and claims 3-6 partially).

SECTION V-----

Claims 1-6 appear to be novel and inventive as the use of rAAV for reducing the immune response to the rAAV or for prolonging expression of a transgene is neither explicitly mentioned nor suggested in the available prior art. Thus, claims 1-6 meet the requirements of Art. 33(2)(3) PCT.

The subject-matter of claim 7 is anticipated by the teaching of Coover D.D. et al., Current Opinion in Neurology, vol. 7, no. 5, October 1994, pp. 463-470 (1) wherein the use of rAAV to deliver and express the dystrophin gene in skeletal muscle cells is described. Although (1) is silent with respect to the absence of a



cytotoxic immune response the disclosure of (1) is considered to be novelty destroying for claim 7 since all steps defined in claim 7 are taught in (1). Thus, carrying out the steps defined in claim 7 the expression of the dystrophin gene must inherently take place in the absence of a cytotoxic immune response. Otherwise, claim 7 would be open to an objection under Art. 6 PCT since at least one feature essential to the performance of the method according to claim 7 would be missing in claim 7.

Thus, claim 7 does not comply with the requirements of Art. 33(2)(3) PCT.

Claims 8-10 can be considered to be novel as the embodiments thereof are not taught in the available prior art. However, considering that the use of helper-free stocks of rAAV to deliver therapeutic genes into cells in general as well as into skeletal muscle cells is already taught in the prior art (see e.g. (1) and WO 95/34670 (2) and US-A-5 436 146 (3)) the subject-matter of claims 8-10 cannot be considered to be inventive but merely as obvious embodiment to a person skilled in the art which arises out of the knowledge of the prior art (Art. 33(3) PCT).

#### SECTION VI-----

WO 97/26337

24.07.97

17.01.97

18.01.96

16.01.97

Herzog et al., PNAS, vol. 94, May 1997, pp. 5804-5809

Fisher et al., Nature Medicine, vol. 3, no. 3, March 1997, pp. 306-313

Kessler et al., PNAS, vol. 93, November 1996, pp. 14082-14087

WO 97/12050

03.04.97

26.09.96

27.09.95

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/US97/15692

SECTION VIII-----

- 1). In the absence of a reference point the terms "reducing" and "prolonging" used in claims 1 and 2, respectively, are vague and thus render the scope of said claims unclear (Art. 6 PCT).
- 2). For the assessment of the present claims 7-10 on the question whether they are industrially applicable, no unified criteria exist in the PCT. The patentability can also be dependent upon the formulation of the claims. The EPO, for example does not recognize as industrially applicable the subject-matter of the claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

## PATENT COOPERATION TREATY

REC'D 17 DEC 1998

WIPO

PCT

## PCT

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference GNVPN.019CIP2PCT	<b>FOR FURTHER ACTION</b>	See Notification of Transmittal of International Preliminary Examination Report (PCT/IPEA/416)
International application No. PCT/US97/15692	International filing date (day/month/year) 04/09/1997	Priority date (day/month/year) 06/09/1996
International Patent Classification (IPC) or national classification and IPC C12N15/86		
Applicant TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.



2. This REPORT consists of a total of 7 sheets, including this cover sheet.

- ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☒ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☒ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand 02/04/1998	Date of completion of this report 15. 12. 98
Name and mailing address of the IPEA/  European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Authorized officer SCHEFFZYK, I Telephone No. (+49-89) 2399-8602 

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/US97/15692

**I. Basis of the report**

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

**Description, pages:**

1-53 as originally filed

**Claims, No.:**

1-10 as originally filed

**Drawings, sheets:**

1/16-16/16 as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:  
☐ the claims, Nos.:  
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

**IV. Lack of unity of invention**

1. In response to the invitation to restrict or pay additional fees the applicant has:

- ☐ restricted the claims.  
☐ paid additional fees.  
☐ paid additional fees under protest.  
☐ neither restricted nor paid additional fees.

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/US97/15692

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.
3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is
- ☐ complied with.
- ☒ not complied with for the following reasons:

**see separate sheet**

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:
- ☒ all parts.
- ☐ the parts relating to claims Nos. .

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Yes:	Claims	1-6, 8-10
	No:	Claims	7
Inventive step (IS)	Yes:	Claims	1-6
	No:	Claims	7-10
Industrial applicability (IA)	Yes:	Claims	1-6
	No:	Claims	7-10: see section VIII/2),

2. Citations and explanations

**see separate sheet**

**VI. Certain documents cited**

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

**see separate sheet**

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/US97/15692

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**VIII. Certain observations on the international application**

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

**see separate sheet**

SECTION IV-----

The common inventive concept underlying the present claims can be seen in the use of adeno-associated virus (rAAV) which is free of contamination with a helper virus for therapeutic purposes. However, the usefulness of such a virus for therapeutic gene delivery to cells, such as for instance skeletal muscle cells, is already taught in the prior art. (see e.g. Coover D.D. et al., Current Opinion in Neurology, vol. 7, no. 5, October 1994, pp. 463-470 (1)).

Thus, the common inventive concept underlying the present claims no longer exists.

Correspondingly, the IPEA is of the opinion that present claims do not relate to one but to two different inventions which are not linked by a common inventive concept as required by Rule 13.1-13.3 PCT, namely:

invention 1: use of rAAV for reducing the immune response to the rAAV when administered into skeletal muscle cells (claims 1, 7-10, complete and claims 3-6, partly) and

invention 2: use of rAAV for prolonging expression of the transgene when administered into skeletal muscle cells (claim 2 complete and claims 3-6 partially).

SECTION V-----

Claims 1-6 appear to be novel and inventive as the use of rAAV for reducing the immune response to the rAAV or for prolonging expression of a transgene is neither explicitly mentioned nor suggested in the available prior art. Thus, claims 1-6 meet the requirements of Art. 33(2)(3) PCT.

The subject-matter of claim 7 is anticipated by the teaching of Coover D.D. et al., Current Opinion in Neurology, vol. 7, no. 5, October 1994, pp. 463-470 (1) wherein the use of rAAV to deliver and express the dystrophin gene in skeletal muscle cells is described. Although (1) is silent with respect to the absence of a

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/US97/15692

cytotoxic immune response the disclosure of (1) is considered to be novelty destroying for claim 7 since all steps defined in claim 7 are taught in (1). Thus, carrying out the steps defined in claim 7 the expression of the dystrophin gene must inherently take place in the absence of a cytotoxic immune response. Otherwise, claim 7 would be open to an objection under Art. 6 PCT since at least one feature essential to the performance of the method according to claim 7 would be missing in claim 7.

Thus, claim 7 does not comply with the requirements of Art. 33(2)(3) PCT.

Claims 8-10 can be considered to be novel as the embodiments thereof are not taught in the available prior art. However, considering that the use of helper-free stocks of rAAV to deliver therapeutic genes into cells in general as well as into skeletal muscle cells is already taught in the prior art (see e.g. (1) and WO 95/34670 (2) and US-A-5 436 146 (3)) the subject-matter of claims 8-10 cannot be considered to be inventive but merely as obvious embodiment to a person skilled in the art which arises out of the knowledge of the prior art (Art. 33(3) PCT).

SECTION VI-----

WO 97/26337	24.07.97	17.01.97	18.01.96
			16.01.97

Herzog et al., PNAS, vol. 94, May 1997, pp. 5804-5809

Fisher et al., Nature Medicine, vol. 3, no. 3, March 1997, pp. 306-313

Kessler et al., PNAS, vol. 93, November 1996, pp. 14082-14087

WO 97/12050	03.04.97	26.09.96	27.09.95
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**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/US97/15692

SECTION VIII-----

- 1). In the absence of a reference point the terms "reducing" and "prolonging" used in claims 1 and 2, respectively, are vague and thus render the scope of said claims unclear (Art. 6 PCT).
  
- 2). For the assessment of the present claims 7-10 on the question whether they are industrially applicable, no unified criteria exist in the PCT. The patentability can also be dependent upon the formulation of the claims. The EPO, for example does not recognize as industrially applicable the subject-matter of the claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

## PATENT COOPERATION TREATY

PCT

## NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

United States Patent and Trademark  
Office  
(Box PCT)  
Crystal Plaza 2  
Washington, DC 20231  
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

<b>Date of mailing (day/month/year)</b> 22 April 1998 (22.04.98)	
<b>International application No.</b> PCT/US97/15692	<b>Applicant's or agent's file reference</b> GNVPN.019CIP2PCT
<b>International filing date (day/month/year)</b> 04 September 1997 (04.09.97)	<b>Priority date (day/month/year)</b> 06 September 1996 (06.09.96)
<b>Applicant</b> WILSON, James, M. et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:

02 April 1998 (02.04.98)

☐ in a notice effecting later election filed with the International Bureau on:2. The election ☒ was☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

<b>The International Bureau of WIPO</b> 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer C. Carrié Telephone No.: (41-22) 338.83.38
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# PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>GNVPN.019CIP2PCT</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/US 97/ 15692</b>	International filing date (day/month/year) <b>04/09/1997</b>	(Earliest) Priority Date (day/month/year) <b>06/09/1996</b>
Applicant <b>TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA et al.</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. ☒ Certain claims were found unsearchable (see Box I).

2. ☐ Unity of invention is lacking (see Box II).

3. ☒ The international application contains disclosure of a nucleotide and/or amino acid sequence listing and the international search was carried out on the basis of the sequence listing

☒ filed with the international application.

☐ furnished by the applicant separately from the international application,

☐ but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.

☐ Transcribed by this Authority

4. With regard to the title, ☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the abstract,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is:

Figure No.            ☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☒ None of the figures.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 97/15692

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

Remark : Although claims 7-10 partially, in so far as they relate to in vivo methods, are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/86 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 34670 A (CHILDRENS HOSPITAL INC) 21 December 1995 see page 5, line 16 - page 9, line 12 see especially page 9, first line see examples 4-7	1-10
X	COOVERT D D ET AL: "GENE THERAPY FOR MUSCLE DISEASES" CURRENT OPINION IN NEUROLOGY, vol. 7, no. 5, October 1994, pages 463-470, XP000673519 see the whole document	1-10
A	US 5 436 146 A (SHENK THOMAS E ET AL) 25 July 1995 see page 5, line 55 - page 6, line 13 see column 9, line 47 - line 62 -/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

2 February 1998

Date of mailing of the international search report

24.03.98

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Sitch, W

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SAMULSKI R J ET AL: "HELPER-FREE STOCKS OF RECOMBINANT ADENO-ASSOCIATED VIRUSES NORMAL INTEGRATION DOES NOT REQUIRE VIRAL GENE EXPRESSION" JOURNAL OF VIROLOGY, vol. 63, no. 9, 1 September 1989, pages 3822-3828, XP000283159 see page 3822 see abstract	
P,X	--- WO 97 26337 A (AVIGEN INC ;UNIV JOHNS HOPKINS (US)) 24 July 1997 see page 6, line 9 - page 9, line 5 see page 20, line 2 - page 34, line 5	1-10
P,X	--- HERZOG ET AL: "STABLE GENE TRANSFER AND EXPRESSION OF HUMAN BLOOD COAGULATION FACTOR IX AFTER INTRAMUSCULAR INJECTION OF RECOMBINANT ADENO-ASSOCIATED VIRUS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES,USA, vol. 94, May 1997, pages 5804-5809, XP002054071 see the whole document	1-10
P,X	--- FISHER ET AL: "RECOMBINANT ADENO-ASSOCIATED VIRUS FOR MUSCLE DIRECTED GENE THERAPY" NATURE MEDICINE, vol. 3, no. 3, March 1997, pages 306-312, XP002054072 see the whole document	1-10
P,X	--- KESSLER ET AL: "GENE DELIVERY TO SKELETAL MUSCLE RESULTS IN SUSTAINED EXPRESSION AND SYSTEMIC DELIVERY OF A THERAPEUTIC PROTEIN" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES,USA, vol. 93, November 1996, pages 14082-14087, XP002054073 see the whole document	1-10
P,X	--- WO 97 12050 A (UNIV ROCKEFELLER ;KAPLITT MARTIN J (US); DIETRICH EDWARD B (US)) 3 April 1997 see page 5, line 29 - page 9, line 21 see page 26; example 1 -----	1-10

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/15692

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9534670 A	21-12-95	US 5658785 A AU 3124395 A CA 2192215 A EP 0764213 A	19-08-97 05-01-96 21-12-95 26-03-97
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WO 9712050 A	03-04-97	AU 7246796 A	17-04-97



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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>A61K 48/00</b>	<b>A2</b>	(11) International Publication Number: <b>WO 98/09657</b> (43) International Publication Date: 12 March 1998 (12.03.98)														
<p>(21) International Application Number: PCT/US97/15692</p> <p>(22) International Filing Date: 4 September 1997 (04.09.97)</p> <p>(30) Priority Data:</p> <table border="0"><tr><td>08/708,188</td><td>6 September 1996 (06.09.96)</td><td>US</td></tr><tr><td>08/729,061</td><td>10 October 1996 (10.10.96)</td><td>US</td></tr></table> <p>(60) Parent Applications or Grants</p> <p>(63) Related by Continuation</p> <table border="0"><tr><td>US</td><td>08/708,188 (CIP)</td></tr><tr><td>Filed on</td><td>6 September 1996 (06.09.96)</td></tr><tr><td>US</td><td>08/729,061 (CIP)</td></tr><tr><td>Filed on</td><td>10 October 1996 (10.10.96)</td></tr></table> <p>(71) Applicant (<i>for all designated States except US</i>): TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; Suite 300, 3700 Market Street, Philadelphia, PA 19104-3147 (US).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (<i>for US only</i>): WILSON, James, M. [US/US]; 1350 N. Avignon Drive, Gladwyne, PA 19035 (US). FISHER, Krishna, J. [US/US]; 2338 Nashville Avenue, New Orleans, LA 70115 (US).</p>		08/708,188	6 September 1996 (06.09.96)	US	08/729,061	10 October 1996 (10.10.96)	US	US	08/708,188 (CIP)	Filed on	6 September 1996 (06.09.96)	US	08/729,061 (CIP)	Filed on	10 October 1996 (10.10.96)	<p>(74) Agents: KODROFF, Cathy, A. et al.; Howson &amp; Howson, Spring House Corporate Center, P.O. Box 457, Spring House, PA 19477 (US).</p> <p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>Without international search report and to be republished upon receipt of that report.</i></p>
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(54) Title: METHOD FOR RECOMBINANT ADENO-ASSOCIATED VIRUS-DIRECTED GENE THERAPY

## (57) Abstract

A method of prolonging gene expression by reducing immune response to a recombinant adeno-associated virus (AAV) bearing a desired gene administered into the muscle of a mammal is described.

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## METHOD FOR RECOMBINANT ADENO-ASSOCIATED VIRUS-DIRECTED GENE THERAPY

This work was supported by the National Institutes  
of Health Grant No. DK47757. The U. S. Government has  
5 certain rights in this invention.

### Background of the Invention

Adeno-associated virus (AAV) is a replication-  
deficient parvovirus, the genome of which is about 4.6 kb  
in length, including 145 nucleotide inverted terminal  
10 repeats (ITRs). The single-stranded DNA genome of AAV  
contains genes responsible for replication (rep) and  
formation of virions (cap).

When this nonpathogenic human virus infects a human  
cell, the viral genome integrates into chromosome 19  
15 resulting in latent infection of the cell. Production of  
infectious virus and replication of the virus does not  
occur unless the cell is coinfecting with a lytic helper  
virus such as adenovirus or herpesvirus. Upon infection  
with a helper virus, the AAV provirus is rescued and  
20 amplified, and both AAV and helper virus are produced.

AAV possesses unique features that make it  
attractive as a vector for delivering foreign DNA to  
cells. Various groups have studied the potential use of  
AAV in the treatment of disease states.

25 Studies of recombinant AAV (rAAV) *in vitro* have been  
disappointing because of low frequencies of transduction;  
incubation of cells with rAAV in the absence of  
contaminating wild-type AAV or helper adenovirus is  
associated with little recombinant gene expression [D.  
30 Russell et al, Proc. Natl. Acad. Sci. USA, 91:8915-8919  
(1994); I. Alexander et al, J. Virol., 68:8282-8287  
(1994); D. Russell et al, Proc. Natl. Acad. Sci. USA,  
92:5719-5723 (1995); K. Fisher et al, J. Virol., 70:520-  
532 (1996); and F. Ferrari et al, J. Virol., 70:3227-3234  
35 (1996)]. Furthermore, integration is inefficient and not

directed to chromosome 19 when rep is absent [S. Kumar et al, J. Mol. Biol., 222:45-57 (1991)]. AAV transduction is substantially enhanced in the presence of adenovirus because the single-stranded rAAV genome is converted to a non-integrated, double-stranded intermediate which is transcriptionally active [K. Fisher et al, J. Virol., 70:520-532 (1996); and F. Ferrari et al, J. Virol., 70:3227-3234 (1996)]. Adenovirus augments rAAV transduction through the expression of the early gene product E4 ORF6 [K. Fisher et al, J. Virol., 70:520-532 (1996); and F. Ferrari et al, J. Virol., 70:3227-3234 (1996)].

The performance of rAAV as a vector for *in vivo* models of gene therapy has been mixed. The most promising results have been in the central nervous system, where stable transduction has been achieved in postmitotic cells [M. Kaplitt et al, Nat. Genet., 8:148-154 (1994)]. Incubation of bone marrow cells *ex vivo* with rAAV results in some transduction, although stable and efficient hematopoietic engraftment has not been demonstrated in transplant models [J. Miller et al, Proc. Natl. Acad. Sci. USA, 91:10183-10187 (1994); G. Podsakoff et al, J. Virol., 68:5656-5666 (1994); and C. Walsh et al, J. Clin. Invest., 94:1440-1448 (1994)]. Administration of rAAV into the airway or the blood leads to gene transfer into lung epithelial cells [K. Fisher et al, J. Virol., 70:520-532 (1996); and T. Flotte et al, Proc. Natl. Acad. Sci. USA, 90:10613-10617 (1993)] and hepatocytes [K. Fisher et al, J. Virol., 70:520-532 (1996)] respectively; however, transgene expression has been found to be low unless adenovirus is present [K. Fisher et al, J. Virol., 70:520-532 (1996)].

What is needed is a method of improving rAAV-mediated gene transfer.

### Summary of the Invention

The present invention provides a method of improving expression of a selected gene delivered to an animal via recombinant AAV. The method involves introducing a  
5 recombinant AAV vector comprising a desired transgene into a muscle cell in the absence of a helper virus. The vector may be administered into cardiac, smooth, or, preferably, skeletal muscle.

In one preferred embodiment, the rAAV-delivered  
10 transgene encodes a secretable and/or diffusable product which is therapeutically useful. In this embodiment, the transgene product may have therapeutic effect on sites at a distance from the delivery site. In another embodiment, the transgene encodes a non-secretable  
15 product (e.g., a dystrophin polypeptide) for which delivery to the muscle is desired (e.g., for treatment of muscular dystrophy).

In another aspect, the present invention provides a method of treating an animal with hemophilia. The method  
20 involves administering into the muscle of the animal a recombinant adeno-associated virus comprising the gene encoding factor IX and sequences which regulate expression of the gene.

In yet another aspect, the invention provides a  
25 method of treating an animal with atherosclerosis. The method involves administering into the muscle of the animal a recombinant adeno-associated virus comprising the gene encoding ApoE and regulatory sequences capable of expressing said gene.

30 Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

### Brief Description of the Drawings

Fig. 1 is a schematic illustration showing the linear arrangement of AAV.CMVlacZ (4883 bp). The relevant elements include AAV ITRs (solid black boxes), CMV promoter (hatched arrow), SV40 intron and polyadenylation signal (open boxes), and lacZ DNA (shaded box). The location of a cDNA probe that can be used to detect the internal BamHI fragment, as well as full-length vector, is also shown.

10 Fig. 2A is a schematic illustration of the linear arrangement of AAV.CMVlacZ concatomer. The relevant landmarks include AAV ITRs (hatched boxes), CMV enhancer/promoter (solid black arrow), SV40 intron and polyadenylation signal (open boxes), and lacZ cDNA (shaded box). AAV.CMVlacZ monomer is shown joined according to a direct end-to-end ligation mechanism (labeled j) at the ITRs. Therefore, in the cartoon two copies of the AAV ITR are present at the junction.

20 Fig. 2B is a schematic illustration showing an amplified view of the junction domain. Relevant landmarks are as indicated in Fig. 3A above. Horizontal arrows indicate the location and direction of PCR primers used to amplify across the provirus junction. Primer 005 is a sense-strand primer. Primers 013 and 017 are antisense-strand primers.

25 Fig. 3 is a schematic illustration showing the predicted PCR product assuming a direct end-to-end tandem ligation of monomer AAV.CMVlacZ genomes. Two complete ITRs (shaded box) with their respective "FLOP" and "FLIP" orientation are shown at the junction (labeled j). The CMV promoter (solid black box) and polyadenylation signal (open box) are also indicated. The PCR cloning site in pCRII is flanked by EcoRI sites as shown. The location of three SnaBI sites positioned within the PCR product are also shown. Primers 005 and 013 also indicated.

30

35

Fig. 4A, in conjunction with Figs. 4B-4G, illustrates the structure of PCR products that map to the head-to-tail junction of AAV.CMVlacZ concatamers of Fig. 4A. Fig. 4A shows the predicted PCR product assuming a direct end-to-end tandem ligation of monomer AAV.CMVlacZgenomes. Two complete ITRs (shaded box) with their respective "FLOP" and "FLIP" orientation are shown at the junction (labeled j). The CMV promoter (solid black box) and polyadenylation signal (open box) are also indicated.

Fig. 4B illustrates the structure of the PCR product from Clone 3.

Fig. 4C illustrates the structure of the PCR product from Clone 8. Clone 8 is nearly identical in size to clone 3, but contains a different rearrangement of the ITR junction.

Fig. 4D illustrates the structure of the PCR product from Clone 5.

Fig. 4E illustrates the structure of the PCR product from Clone 2.

Fig. 4F illustrates the structure of the PCR product from Clone 6.

Fig. 4G illustrates the structure of the PCR product from Clone 7.

Fig. 5A characterizes the activation of cytotoxic T lymphocytes directed against adenoviral antigen as well as lacZ. This is an analysis of lymphocytes harvested from Group 1 of Example 5.

Fig. 5B characterizes the activation of cytotoxic T lymphocytes directed against adenoviral antigen as well as lacZ. This is an analysis of lymphocytes harvested from Group 2 of Example 5.

Fig. 5C characterizes the activation of cytotoxic T lymphocytes directed against adenoviral antigen as well

as lacZ. This is an analysis of lymphocytes harvested from Group 3 of Example 5.

Fig. 6A shows the activation of T lymphocytes in response to different antigens including  $\beta$ -galactosidase, purified AAV, or adenovirus, for each of Groups 1-4 of Example 5. Activation is demonstrated by the secretion of IFN- $\gamma$  representing the TH1 subset of T cells.

Fig. 6B shows the activation of T lymphocytes in response to different antigens including  $\beta$ -galactosidase, purified AAV, or adenovirus, for each of Groups 1-4 of Example 5. Activation is demonstrated by the secretion of IL-10 representing the TH2 subset of T cells.

Fig. 7A illustrates results from an enzyme linked immunosorbent assay (ELISA), showing the development of antibodies directed against  $\beta$ -galactosidase in the various groups of example 5.

Fig. 7B illustrates results from an enzyme linked immunosorbent assay (ELISA), showing the development of antibodies directed against adenovirus type 5 in the various groups of Example 5.

Fig. 8 is a graph of plasma concentration of hF.IX in C57BL/6 mice as a function of time following IM injection of  $2 \times 10^{11}$  rAAV-hF.IX vector genomes/animal (n=4).

Fig. 9 is a graph showing circulating antibody against human F.IX as a result of im injection of rAAV-hF.IX in C57BL/6 mice. The time course of anti-hF.IX antibody concentration in plasma after injection with  $2 \times 10^{11}$  vector genomes/animal (n=3) was determined by ELISA using mouse MAb anti-hF.IX [Boehringer Mannheim] as a standard. Each line represents an individual animal.

Fig. 10 illustrates plasma concentration of hF.IX in three mice as a function of time post-injection. Each symbol represents a different animal. The fourth animal



in this experiment died 5 weeks post-injection following traumatic phlebotomy.

Fig. 11 is a graph illustrating plasma concentration of hF.IX in four Rag-1 mice as a function of time post-injection with rAAV-hF.IX. Each symbol represents a different animal.

Fig. 12 is a schematic diagram of a head-to-tail concatamer of the rAAV present in transduced cells.

Fig. 13 is a schematic diagram illustrating the construction of AV.CMVApoE.

#### Detailed Description of the Invention

The present invention provides a method for adeno-associated virus (AAV) mediated muscle-directed gene transfer which provides high level and stable transgene expression in the absence of helper virus or exogenous helper molecules. Particularly, this method involves introducing a recombinant AAV carrying a desired transgene into a muscle cell. Desirably, the rAAV vector is injected directly into cardiac, skeletal, or smooth muscle and the transgene encodes a secreted and/or diffusable therapeutic product, such as a polypeptide or an RNA molecule. However, the method of the invention is similarly useful for administration of nucleic acids encoding non-secreted, therapeutically useful products.

Particularly, the inventors have discovered that intramuscular injection of helper-free purified rAAV (i.e., rAAV which is substantially free of contamination with adenovirus or wild-type AAV) leads to highly efficient transduction of muscle fibers leading to stable and prolonged transgene expression. By helper-free is further meant that the AAV is in the substantial absence of helper virus or other exogenous helper molecules (i.e., helper molecules not native to or normally present in muscle cells). This is accomplished without

significant inflammation or activation of immune responses to the transgene product, despite the fact that the product may be a neoantigen.

The stability of transgene expression produced according to the methods of this invention is particularly impressive. Without wishing to be bound by theory, this stability is believed to be due to the highly inefficient chromosomal integration of AAV proviral DNA in muscle cells in the absence of helper virus or other exogenous helper molecules. Several observations support this hypothesis. As described in the examples below, analysis of Hirt extracts failed to detect a double-stranded episomal form of the viral genome. Southern analysis of total cellular DNA revealed a discrete band when digested with an enzyme that has two cleavage sites within the AAV vector, whereas no discrete band was observed when the same DNA was digested with an enzyme that does not have sites within the viral genome.

Further DNA analysis focused on the formation of concatamers and their structure. Previous studies of lytic AAV infections have shown that the episomal replication of rAAV proceeds through head-to-head or tail-to-tail concatamers, while latent infections that result in proviral integration are established as head-to-tail tandem arrays [K.I. Berns, Microbiol. Rev., 54:316-329 (1990); J.-D. Tratschin et al, Mol. Cell. Biol., 5:3251-3260 (1985); N. Muzczynska, Current Topics in Microbiology and Immunology, 158:97-129 (1993); S.K. McLaughlin et al, J. Virol., 62:1963-1973 (1988)]. The data set forth herein demonstrate that muscle cells transduced with rAAV vectors according to this invention contain concatamers consisting of head-to-tail tandem arrays with variable deletions of both ITRs, consistent with a transduction mechanism involving integration of rAAV provirus.

Sequence analysis of the junctions created by rAAV concatamers indicated consistent but variable deletions of both ITRs. Fluorescent in situ hybridization (FISH) analysis was consistent with single integration sites in approximately 1 in 20 nuclei, while Southern analysis indicated an average of 1 proviral genome per diploid genome of the muscle fiber. Together these findings indicate that the average concatamers minimally comprises ten proviral genomes.

Another advantage of the method of the invention is the surprising absence of inflammation upon administration of therapeutic doses of vector. For example, C57BL/6 mice injected with a lacZ AAV vector failed to mount a humoral immune response to *E.coli*  $\beta$ -galactosidase despite the fact that vibrant anti- $\beta$ -galactosidase antibodies were elicited in these animals when a lacZ adenoviral vector was injected into skeletal muscle. Thus, when the helper-free AAV vector was used according to the method of this invention, immune responses to the transgene were modulated. This contrasts sharply with prior art methods of gene transfer, such as those which employ naked plasmid DNA [J.A. Wolff et al, Science, 247:1465-1468 (1990)] or adenovirus-mediated gene therapy [S.K. Tripathy et al, Nat. Med., 2:545-550 (1996)], which typically elicit strong immune responses to the transgene. Thus, the method of the invention provides a significant advantage over other gene delivery systems, particularly with respect to the treatment of chronic disorders that may require repeated administrations.

#### I. The Recombinant AAV

A recombinant AAV vector carrying a selected transgene is used in the methods of the invention. In addition to the transgene, the vector further contains

regulatory sequences which control expression of the transgene in a host cell, e.g., a muscle cell.

Many rAAV vectors are known to those of skill in the art and the invention is not limited to any particular rAAV vector. For example, suitable AAV vectors and methods of producing same are described in U. S. Patent No. 5,252,479; U. S. Patent No. 5,139,941; International Patent Application No. WO94/13788; and International Patent Application No. WO93/24641. One particularly desired vector is described below.

A. The AAV Sequences

Currently, a preferred rAAV is deleted of all viral open reading frames (ORFs) and retains only the cis-acting 5' and 3' inverted terminal repeat (ITR) sequences [See, e.g., B. J. Carter, in "Handbook of Parvoviruses", ed., P. Tijsser, CRC Press, pp.155-168 (1990)]. Thus, the rep and cap polypeptide encoding sequences are deleted. The AAV ITR sequences are about 143 bp in length. While it is preferred that substantially the entire 5' and 3' sequences which comprise the ITRs are used in the vectors, the skilled artisan will understand that some degree of minor modification of these sequences is permissible. The ability to modify these ITR sequences while retaining their biological functions is within the skill of the art. See, e.g., texts such as Sambrook et al, "Molecular Cloning. A Laboratory Manual.", 2d edit., Cold Spring Harbor Laboratory, New York (1989).

The AAV ITR sequences may be obtained from any known AAV, including presently identified human AAV types. The selection of the AAV type is not anticipated to limit the invention. A variety of AAV types, including types 1-4, are available from the American Type Culture Collection or available by request from a variety of commercial and institutional sources. Similarly, AAVs

known to infect other animals may also be employed in the vector used in the methods of this invention. In the examples set forth herein, an AAV-2 is used for convenience. Specifically, the 5' and 3' AAV ITR sequences from AAV-2 flank a selected transgene sequence and associated regulatory elements, as described below.

B. The Transgene

The transgene sequence contained within the rAAV vector is a nucleic acid sequence, heterologous to the AAV sequence, which encodes an RNA or polypeptide of interest. The transgene is operatively linked to regulatory components in a manner which permits transgene expression in muscle cells.

The composition of the transgene sequence will depend upon the use to which the resulting vector will be put. For example, one type of transgene sequence includes a reporter sequence, which upon expression produces a detectable signal. Such reporter sequences include without limitation an *E. coli* beta-galactosidase (*LacZ*) cDNA, an alkaline phosphatase gene and a green fluorescent protein gene. These sequences, when associated with regulatory elements which drive their expression, provide signals detectable by conventional means, e.g., ultraviolet wavelength absorbance, visible color change, etc. Expression of such transgenes may be used in methods for cell quantitation, cell identification and the like.

A more preferred transgene sequence includes a therapeutic gene which encodes a desired gene product. These therapeutic nucleic acid sequences typically encode products which, when administered to a patient *in vivo* or *ex vivo*, are able to replace or correct an inherited or non-inherited genetic defect or treat an epigenetic disorder or disease.

The method of the invention, which delivers the transgene to the muscle cells, is particularly well suited for use in connection with secreted therapeutic proteins, such as factor IX, useful in treatment of hemophilia, or apolipoprotein (Apo) E, useful in treatment of atherosclerosis. However, other therapeutic gene products, particularly those which are secreted, may be readily selected by the skilled artisan. Examples of genes encoding secreted and/or diffusable products, include, without limitation, cytokines, growth factors, hormones, differentiation factors, and the like, e.g.,  $\beta$ -interferon ( $\beta$ -IFN), erythropoietin (epo), insulin, growth hormone (GH), and parathyroid hormone (PTH). These genes are useful for treatment of a variety of conditions, including multiple sclerosis and cancer ( $\beta$ -IFN), anemia (epo), diabetes (insulin), small stature (GH), and osteoporosis (PTH). The method of the invention is also useful for delivery of genes encoding non-secreted products to the muscle. For example, the method of the invention is anticipated to be useful in treatment of muscular dystrophies, by enabling delivery of a dystrophin gene [see, e.g., C. C. Lee et al, Nature, 349:334-336 (1991)] via a rAAV according to the method of the invention. The selection of the transgene is not considered to be a limitation of this invention, as such selection is within the knowledge of the skilled artisan.

### C. Regulatory Elements of the Vector

In addition to the AAV ITR sequences and the transgene, the vector also includes regulatory elements necessary to drive expression of the transgene in transduced muscle cells. Thus the vector desirably contains a selected promoter and enhancer (if desired) which is operatively linked to the transgene and located, with the transgene, between the AAV ITR sequences of the vector.

Selection of the promoter and, if desired, the enhancer, is a routine matter and is not a limitation of the vector itself. Useful promoters may be constitutive promoters or regulated (inducible) promoters, which will enable controlled expression of the transgene. For example, a desirable promoter is that of the cytomegalovirus immediate early promoter/enhancer [see, e.g., Boshart et al, Cell, 41:521-530 (1985)]. Other desirable promoters include, without limitation, the Rous sarcoma virus LTR promoter/enhancer and the inducible mouse metallothienien promoter. Still other promoter/enhancer sequences may be selected by one of skill in the art.

The vectors will also desirably contain nucleic acid sequences which affect transcription or translation of the transgene including sequences providing signals required for efficient polyadenylation of the transcript and introns with functional splice donor and acceptor sites. A common poly-A sequence which is employed in the exemplary vectors of this invention is that derived from the papovavirus SV-40. The poly-A sequence generally is inserted into the vector following the transgene sequences and before the 3' AAV ITR sequence. A common intron sequence is also derived from SV-40, and is referred to as the SV-40 T intron sequence. Selection of these and other elements desirable to control or enhance gene expression are conventional and many such sequences are known to those of skill in the art [see, e.g., Sambrook et al, and references cited therein].

The combination of the transgene, promoter/enhancer and the other regulatory elements are referred to as a "minigene" for ease of reference herein. As above stated, the minigene is flanked by the 5' and 3' AAV ITR sequences. Provided with the teachings of this

invention, the design of such a minigene can be readily accomplished by the skilled artisan.

An example of a rAAV, i.e., AAV.CMVLacZ, and its use in the method of the invention is provided in the examples below. As illustrated in Fig. 1, this exemplary rAAV contains a 5'AAV ITR, a CMV promoter, an SV-40 intron, a LacZ transgene, an SV-40 poly-A sequence and a 3'AAV ITR. However, as stated above, the method of this invention is not limited to use of any particular rAAV.

10           D.   Production of rAAV

The sequences employed in the construction of the rAAV used with the method of this invention may be obtained from commercial or academic sources based on previously published and described materials. These materials may also be obtained from an individual patient or generated and selected using standard recombinant molecular cloning techniques known and practiced by those skilled in the art. Any modification of existing nucleic acid sequences used in the production of the rAAV vectors, including sequence deletions, insertions, and other mutations may also be generated using standard techniques.

Assembly of the rAAV, including the sequences of AAV, the transgene and other vector elements, may be accomplished using conventional techniques. One particularly desirable technique is described in K. J. Fisher et al, J. Virol., 70(1):520-532 (January, 1996), which is incorporated by reference herein. However, other suitable techniques include cDNA cloning such as those described in texts [Sambrook et al, cited above], use of overlapping oligonucleotide sequences of the AAV genome, polymerase chain reaction, and any suitable method which provides the desired nucleotide sequence. Where appropriate, standard transfection and co-transfection techniques are employed to propagate the



rAAV viruses in the presence of helper viruses, e.g., adenoviruses deleted for E1 using techniques such as CaPO<sub>4</sub> transfection techniques, and may be readily selected by the skilled artisan. Other conventional  
5 methods which may be employed in this invention include homologous recombination of AAV viral genomes, plaquing of viruses in agar overlay, methods of measuring signal generation, and the like.

Desirably, the rAAV produced are purified to  
10 remove any contaminating adenovirus or wild-type AAV. A particularly desirable purification scheme is described in K. J. Fisher et al, J. Virol., 70(1):520-532 (January, 1996), which is incorporated by reference. However, one of skill in the art can readily select other appropriate  
15 purification means.

## II. Therapeutic Applications

Once a rAAV containing a desired transgene is obtained, the vector is administered directly into an animal's muscle. One advantage of the method of the  
20 invention is that muscle is particularly well suited as a site for production of secreted therapeutic products, such as factor IX or apolipoprotein (Apo) E, among others. Alternatively, the method of the invention is used to deliver a non-secreted gene product to the muscle  
25 cells.

The rAAV vectors of the present invention may be administered to a patient, preferably suspended in a biologically compatible solution or pharmaceutically acceptable carrier or delivery vehicle. A suitable  
30 vehicle includes sterile saline. Other aqueous and non-aqueous isotonic sterile injection solutions and aqueous and non-aqueous sterile suspensions known to be pharmaceutically acceptable carriers and well known to

those of skill in the art may be employed for this purpose.

The rAAV vectors of this invention are administered in sufficient amounts to provide for integration and expression of the selected transgene such that a therapeutic benefit may be obtained without undue adverse effects and with medically acceptable physiological effects which can be determined by those skilled in the medical arts. In a preferred embodiment, the rAAV are injected directly into cardiac, skeletal, or smooth muscle. One of skill in the art will also appreciate that other methods of administration, e.g., intravenous or intraarterial injection may also be utilized in the method of the invention so long as the rAAV is targeted to the muscle cells.

Dosages of the rAAV vector will depend primarily on factors such as the condition being treated, the selected transgene, the age, weight and health of the patient, and may thus vary among patients. A therapeutically effective dose of the rAAV of the present invention is believed to be in the range of from about 1 to about 50 ml of saline solution containing concentrations of from about  $1 \times 10^8$  to  $1 \times 10^{11}$  particles/ml rAAV vector of the present invention. Desirably, each dose contains at least  $10^9$  particles rAAV. A more preferred human dosage is about 1-20 ml saline solution at the above concentrations. The levels of expression of the selected transgene can be monitored by bioassay to determine the route, dose or frequency of administration.

Administration of the rAAV may be repeated as needed.

The examples set forth below illustrate the preferred methods for preparing the vectors and performing the methods of the invention. These examples are illustrative only and do not limit the scope of the invention.

Example 1 - Production of AAV.CMVLacZ

A recombinant AAV (rAAV) was generated in which the rep and cap genes were replaced with a minigene expressing *E. coli*  $\beta$ -galactosidase under the control of a CMV promoter (AAV.CMVLacZ). AAV.CMVLacZ was produced by using the cis-acting plasmid pAAV.CMVLacZ, which was derived from psub201 [R. Samulski et al, J. Virol., 61(10):3096-3101 (1987)]. Briefly, the plasmid was transfected into 293 cells infected with E1-deleted adenovirus [K.J. Fisher et al, J. Virol., 70:520-532 (1996)] and AAV rep and cap functions were provided by a transacting plasmid pAAV/Ad [R. Samulski et al, J. Virol., 63:3822-3826 (1989)]. Production lots of AAV.CMVLacZ vector were titered according to genome copies/ml as described [Fisher et al, J. Virol., 70:520-532 (1996)].

The 5'-to-3' organization of the AAV.CMVLacZ genome (4883 bp), includes:

the 5' AAV ITR (bp 1-173) obtained by PCR using pAV2 [C. A. Laughlin et al, Gene, 23: 65-73 (1983)] as template [nucleotide numbers 365-538 of SEQ ID NO: 1];

a CMV immediate early enhancer/promoter [Boshart et al, Cell, 41:521-530 (1985); nucleotide numbers 563-1157 of SEQ ID NO: 1],

an SV40 intron (nucleotide numbers 1178-1179 of SEQ ID NO: 1),

an *E. coli* lacZ cDNA (nucleotide numbers 1356 - 4827 of SEQ ID NO: 1),

an SV40 polyadenylation signal (a 237 Bam HI-BclI restriction fragment containing the cleavage/poly-A signals from both the early and late transcription units; nucleotide numbers 4839 - 5037 of SEQ ID NO: 1) and

the 3' AAV ITR, obtained from pAV2 as a SnaBI-BglII fragment (nucleotide numbers 5053 - 5221 of SEQ ID NO:

1).

With reference to Fig. 1, two Bam HI sites are present in the double-stranded vector sequence. The first is located in the SV40 intron at bp position 875 and the second lies between the lacZ DNA and the SV40 polyadenylation signal at bp position 4469. Therefore, digestion of the double-stranded sequence with BamHI releases a fragment of 3595 bp in length. The location of a cDNA probe that can be used to detect the internal BamHI fragment, as well as full-length vector, is also shown.

The rAAV.CMVlacZ virus was purified using standard techniques [see, e.g., K. F. Kozarsky et al, J. Biol. Chem., 269:13695-13702 (1994)]. Stocks of rAAV used in the following examples were tested to ensure the absence of replication competent wild-type AAV and helper E1-deleted adenovirus as follows.

293 cells were seeded in chamber slides and co-infected with wild-type adenovirus and an aliquot of the rAAV vector stock. Twenty hours post-infection, cells were fixed and incubated with a mouse monoclonal antibody against AAV capsid proteins (American Research Products). Antigen-antibody complex was detected with a FITC-conjugated secondary antibody. A positive signal was scored as one infectious AAV unit. Contaminating helper adenovirus was measured by infecting 293 cells with an aliquot of the rAAV vector stock and staining for alkaline phosphatase reporter expression. The helper adenovirus is E1-deleted and contains a human placenta alkaline phosphatase cDNA under the transcriptional control of the CMV promoter. Replication-competent wild-type AAV or adenovirus helper was not detected in the highly purified preparations of rAAV.

Example 2 - rAAV Stably Transduces Skeletal Muscle in Vivo

rAAV was administered with and without an E2a-deleted adenovirus, which was intended to enhance transduction. Animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Wistar Institute.

Briefly, five week old female C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine) were anesthetized with an intraperitoneal injection of ketamine (70 mg/kg) and xylazine (10 mg/kg) and a subsequent 1 cm lower extremity incision was made. Samples of rAAV.CMVLacZ ( $1 \times 10^9$  vector genomes) in 25  $\mu$ l of HEPES-Buffered-Saline (HBS, pH 7.8) or rAAV.CMVLacZ supplemented with an adenovirus E2a mutant dl802 [S. A. Rice and D. L. Klessig, J. Virol., 56:767-778 (1985)] ( $5 \times 10^{10}$   $A_{260}$  particles,  $1 \times 10^8$  pfu) just prior to injection, were injected into the tibialis anterior muscle of each leg using a Hamilton syringe. Incisions were closed with 4-0 Vicryl suture. To analyze transgene expression, animals were necropsied at various time points post-injection and injected muscle was excised with a scalpel. Tissue was placed on a drop of OTC embedding compound, snap-frozen in liquid nitrogen-cooled isopentane for seven seconds, and immediately transferred to liquid nitrogen. Analysis of tissue at each time point represented a minimum of 6 injection sites (i.e., bilateral sampling from at least 3 animals).

For histochemical analysis, frozen muscle was segmented laterally into two equal halves, generating a cross-section face of the tissue. Both halves of the tissue were serially sectioned (6  $\mu$ m). For X-gal histochemistry, sections were fixed in a freshly prepared 0.5% glutaraldehyde solution in PBS and stained for  $\beta$ -galactosidase activity as described [K.J. Fisher et al,

J. Virol., 70:520-532 (1996)]. Sections were counterstained in neutral red solution and mounted.

When adenovirus was used as a helper for the rAAV, X-gal histochemistry analysis revealed high level  
5 transduction of muscle fibers by day 17 associated with substantial inflammation. Surprisingly, however, animals that received rAAV in the absence of adenovirus helper demonstrated levels of transduction that exceeded those found in the presence of adenovirus. These high levels  
10 have persisted without apparent diminution for 180 days.

Example 3 - rAAV Genome Integrates with High Efficiency as Truncated Head-to-Tail Concatamers

In order to characterize the molecular state of the stabilized rAAV genome, Southern blot analysis of DNA  
15 from skeletal muscle harvested from mice injected as above was performed. Models in which the rAAV genome persists either as an episomal double-stranded genome such as those formed during lytic infection, or as an integrated provirus resembling latent infection were  
20 considered.

Briefly, low molecular weight DNA (Hirt) (see Part A below) and high molecular weight genomic DNA (see Part B below) was isolated from mouse muscle at selected time points. DNA samples were resolved on a 1% agarose gel and  
25 electrophoretically transferred to a nylon membrane (Hybond-N, Amersham). The blot was hybridized with a <sup>32</sup>P-dCTP random-primer-labeled restriction fragment isolated from the lacZ cDNA.

A. Detection of Episomal Double-Stranded Genome

30 To detect nonintegrated forms of the rAAV genome, Hirt extracts of transduced muscle DNA were analyzed by hybridization with a <sup>32</sup>P-labeled cDNA that maps to the probe sequence shown in Fig. 1. The Hirt DNA samples (15 µl, equivalent to 15 mg tissue) were

extracted from muscle harvested on day 8, 17, 30, and 64 post-injection.

DNA from a cultured cell line infected with rAAV in the presence of adenovirus was analyzed. The analysis of Hirt extracts from that cell line demonstrated the presence of both single-stranded and monomeric double-stranded forms of the virus. However, Hirt extracts of muscle transduced with rAAV alone demonstrated the single-stranded genome by day 8 that diminished to undetectable levels by day 64. Double-stranded forms of rAAV were never detected in the Hirt extracts even when the filters were over-exposed. This indicates that the single-stranded rAAV genome is efficiently transferred into cells of skeletal muscle; however, it is not converted to transcriptionally active episomal forms.

#### B. Detection and Characterization of Integrated Proviral DNA

To detect integrated proviral DNA, additional hybridization studies were performed with total cellular DNA harvested from transduced skeletal muscle 64 days post-infection. Genomic DNA (10  $\mu$ g, equivalent to 18  $\mu$ g tissue) was digested with BamHI or HindIII, a restriction enzyme that does not cut proviral DNA. As expected, HindIII digestion resulted in a smear after gel fractionation and hybridization to a virus specific probe. However, when genomic DNA was digested with BamHI, which cuts twice within the provirus, a discrete band of the predicted size of 3.6 kb was detected at an abundance of approximately 1 proviral genome/diploid host cell genome.

The structure of the integrated provirus was characterized using PCR analysis to delineate the potential mechanisms of persistence. Previous studies of wild type and rAAV have suggested different pathways of

DNA replication in the lytic and latent phases of the viral life cycle. Specifically, in the presence of helper virus, AAV replicates to form dimeric replicative intermediates by a mechanism that results in the synthesis of head-to-head or tail-to-tail concatamers. This contrasts with latent infections where the integrated proviral genome is characterized by head-to-tail genomic arrays.

Genomic DNA from skeletal muscle was subjected to PCR analysis to amplify junctions between AAV genomic concatamers. A PCR method to detect integrated rAAV was developed, based on data indicating that integrated forms of rAAV are typically found as head-to-tail concatamers. Specifically, oligonucleotide primers were synthesized to allow selective PCR amplification across head-to-tail junctions of two monomers of the AAV.CMV<sub>LacZ</sub> genome. The sense-strand primer 005 (5'-ATAAGCTGCAATAACAAGT-3'; SEQ ID NO: 4) mapped to bp position 4584-4603 of the SV40 polyadenylation signal domain. The antisense-strand primer 013 (5'-CATGGTAATAGCGATGACTA-3'; SEQ ID NO:2) mapped to bp position 497-478 of the CMV promoter, while the antisense-strand primer 017 (5'-GCTCTGCTTATATAGACCTC-3'; SEQ ID NO:3) mapped to bp position 700-680 of the CMV promoter. If the ITRs are retained intact, oligos 005 + 013 [SEQ ID NO:2] should amplify a 797 bp fragment while oligos 005 and 017 [SEQ ID NO: 3] should amplify a 1000 bp fragment. It is important to emphasize that the predicted PCR product sizes are based on the assumption that provirus junctions contain two ITR copies. Amplification across a junction that has fewer than two copies will therefore generate a PCR product that is proportionally smaller in size.

PCR reactions were performed using 100 ng genomic DNA template and primer concentrations of 0.5  $\mu$ M. The thermocycle profile was 94° C 1 min, 52° C 1 min, and



72° C 1 min 30 sec for 35 cycles; the 94° C denaturation step of the first cycle was 2 min, while the 72° C extension step of the last cycle was 10 min. PCR products were analyzed by agarose gel electrophoresis.

5               PCR reactions were conducted on genomic DNA isolated from AAV.CMVLacZ transduced muscle harvested at day 64 post-infection, as described above. Genomic DNA from muscle injected with Hepes buffered saline (HBS) was used as a negative PCR control. No amplification  
10 products were detected when primers were used that should span a head-to-head or tail-to-tail junction (data not shown). However, when DNA from AAV.CMVLacZ transduced muscle was analyzed with oligonucleotides 005 and 013 and  
15 005 and 017, a smear was detected consistent with a heterogenous population of head-to-tail concatamers (Figs. 2A and 2B).

              PCR reactions were also conducted with genomic DNA from cell lines that contain integrated AAV.CMVLacZ. The provirus structure of these clones has been  
20 determined by Southern blot analysis. Three cell lines (10-3.AV5, 10-3.AV6, and 10-3.AV18) each of which contain at least two monomer copies of integrated AAV.CMVLacZ arranged head-to-tail were identified. Based on the size  
25 of the PCR products (a 720 bp product using primer set 005-013, and a 930 bp product using primer set 005-017), two clones 10-3.AV5 and 10-3.AV6 likely contain 1.5  
copies of AAV ITR at the junction. Another clone 10-3.AV18 contains a large deletion that encompasses the AAV ITRs producing a 320 bp product using primer set 005-013  
30 and a 500 bp product using primer set 005-017. Another cell line 10-3.AV9 contains a single monomer copy of integrated AAV.CMVLacZ according to Southern blotting, and appears to be confirmed by the absence of a PCR product.

Thus, analysis of DNA from rAAV infected cell lines selected for stable transduction revealed distinct bands smaller than that predicted for an intact head-to-tail concatamer.

5           D.    Structural Analysis

Detailed structural analyses of the proviral junctions recovered from skeletal muscle DNA was performed by subcloning from the PCR reaction (Fig. 3) followed by restriction analysis (Figs. 4A-4G).

10   Particularly, PCR products from one of the muscle samples BL.11 obtained as described above were directly ligated into commercially available plasmid pCRII in which the insert was flanked by EcoRI sites. Commercially available competent bacterial strain TOP10 F' were  
15   transformed with the ligation reactions. In effect, this procedure results in a plasmid library of PCR products. The library was plated at a density to give well isolated colonies and screened by overlaying with a nylon membrane and hybridizing with a <sup>32</sup>P-labeled fragment corresponding  
20   to the CMV promoter/enhancer. Putative positive clones were grown overnight in small-scale cultures (2 ml).

Plasmid DNA from six representative clones was extracted from the small-scale cultures and digested with either EcoRI to release the entire PCR product or with  
25   SnaBI as a diagnostic indicator. Digestion with SnaBI should release a 306 bp fragment (SnaBI 476 to SnaBI 782) spanning the CMV promoter. The release of a second fragment mapping to the ITR junction (SnaBI 142 to SnaBI 476) is contingent on rearrangements that occur during  
30   formation of the concatomer, and could therefore range in size from 334 bp (2 complete ITR copies) to 0 bp if the ITRs have been deleted.

The PCR product from cell line 10-3.AV5 believed to contain 1.5 copies of AAV ITR (10-3.AV5) was  
35   also cloned into pCRII and digested with the indicated

enzyme. This sample serves as a positive control for the diagnostic SnaBI digestion. Digestion of this sample with EcoRI correctly releases the 730 bp PCR fragment, as well as a secondary doublet band approximately 500 bp in size. This secondary band is believed to be an artifact due to secondary structure that develops in the 1.5 copies of AAV ITR during replication in bacteria. Digestion of the positive control with SnaBI releases the diagnostic 306 bp fragment from the CMV promoter and a 250 bp fragment that maps to the ITR junction.

Digestion of the six individual clones with EcoRI and SnaBI indicated deletions of variable lengths were present in all recovered junctions and largely confined to ITRs at the junctions. Sequence analyses further indicated that most deletions spanned portions of both ITRs at the junctions without involving contiguous viral DNA.

E. Fluorescence in situ hybridization (FISH)  
Analysis

FISH was performed on cryosections of skeletal muscle to characterize the distribution of proviral DNA within the injected tissue. Small 4-5 mm pieces of muscle from treated or control mice were embedded in OTC and quickly frozen in liquid isopentane cooled with liquid nitrogen. Frozen sections, 10 $\mu$  thick were cut on a cryomicrotome. Sections were mounted, fixed (Histochoice) and processed for fluorescence by in situ hybridization using a previously described protocol [E. Gussoni et al, Nat. Biotech., 14:1012-1016 (1996)]. Adjacent sections were stained for  $\beta$ -galactosidase activity to identify lacZ positive areas in muscle bundles.

For quantifying FISH signal, lacZ positive areas (as determined by staining adjacent sections for  $\beta$ -galactosidase activity) were examined under a Nikon

microphot FxA microscope equipped with epifluorescence. The total number of individual muscle fibers corresponding to *lacZ* positive area in the section were counted under a standard phase contrast mode. The same  
5 area was then examined under fluorescence microscopy using the appropriate filter package for rhodamine isothiocyanate. The number of muscle cell nuclei showing punctate staining was recorded. Each positive nucleus was examined under phase contrast to ascertain that it  
10 came from a muscle fiber. For controls, *lacZ* negative areas (areas in the same sections that lacked  $\beta$ -galactosidase activity, or mock transfected muscle sections) were examined and quantified in a similar manner.

15 For confocal microscopy, sections were observed under an oil immersion objective lens (100X) on a Leica confocal laser microscope equipped with Krypton-Argon laser (Leica lasertechnik, GmbH), TSC and Voxel View Silicon graphics central work stations. Images observed  
20 under rhodamine channel were also sequentially observed under differential interference contrast to confirm the location of fluorescence signal with muscle cell nuclei. The differential contrast and fluorescence images were then sequentially superimposed on a TCS central work  
25 station and were transferred to a Silicon graphics work station for image processing. Processed images were stored and printed using Photoshop software.

Serial sections were alternatively stained for  $\beta$ -galactosidase activity to identify transgene expressing  
30 muscle fibers and hybridized with a biotinylated proviral probe to localize the distribution of the proviral genome. A discrete fluorescent signal was detected in some nuclei of  $\beta$ -galactosidase expressing muscle fibers. A survey of three serial sections revealed hybridization  
35 in 53/1006 (5.3%) nuclei of  $\beta$ -galactosidase expressing

fibers and 0/377 nuclei of fibers not expressing  $\beta$ -galactosidase. Hybridization was not detected in tissues from uninfected animals (data not shown).

The ability to detect viral genomes by FISH added  
5 another dimension to the analysis. Single foci of hybridization was detected in 5% of all nuclei contained within muscle fibers expression  $\beta$ -galactosidase. It is possible that this is an underestimate of transduced nuclei, because of limitations in sensitivity of this  
10 technique especially for target sequences less than 12 kb in size [B.J. Trask, Trends Genet., 7:149-154 (1991)]. The implications of the FISH analysis are interesting. The presence of 1 proviral genome/diploid myoblast genome, as measured by Southern, together with the FISH  
15 result that demonstrated 5% of nucleic acids harboring an AAV genome would predict that the average concatamer minimally comprises ten proviral genomes. These studies show  $\beta$ -galactosidase enzyme activity that extends far beyond the site of a vector transduced nucleus,  
20 suggesting an extended nuclear domain of at least 10  $\mu$ m. This is consistent with previous work which documented extended nuclear domains for cytosolic proteins [H.M. Blau et al, Adv. Exp. Med. & Biol., 280:167-172 (1990)]. An extended nuclear domain of transgene expression within  
25 the syncytial structure of the muscle fibers is important to applications of gene therapy for several reasons. The net yield of recombinant protein from a transduction event may be higher in a syncytium where the distribution of protein is less constrained by membrane barriers.  
30 Furthermore, this system has advantages in vector systems that require expression of multiple recombinant proteins such as those with inducible promoters [J.R. Howe et al, J. Biol. Chem., 270:14168-14174 (1995); V. M. Rivera et al, Nat. Med., 2:1028-1032 (1996)]. In muscle,

coexpression of recombinant proteins does not require cotransduction with a single nucleus because of the extensive network of overlapping domains.

Example 4 - Transgene Directed Immune Responses are  
5 Minimized when Helper-Free rAAV is Used for Muscle-  
Directed Gene Delivery

The stability of lacZ expression in muscle cells achieved from a lacZ-containing rAAV vector administered in the absence of helper virus was surprising in light of  
10 previous work which demonstrated destructive immune responses mounted against  $\beta$ -galactosidase expressed from adenoviral vectors in muscle fibers. Transgene-specific immune responses were studied further by measuring the serum levels of anti- $\beta$ -galactosidase antibodies using  
15 Western analysis.

Blood was drawn from C57BL/6 and ROSA-26 mice (Jackson Laboratories, Bar Harbor, ME) necropsied on day 30 after virus injection and serum was collected. ROSA-26 is a transgenic line that carries the *E. coli*  
20  $\beta$ -galactosidase cDNA and was developed on a 129 background. Serum was also harvested from C57BL/6 mice that received an intramuscular injection of either a recombinant LacZ adenovirus (H5.010CMVLacZ,  $5 \times 10^8$  pfu in 25  $\mu$ l of HBS) or the recombinant AAV.CMVLacZ (as  
25 described above). Both vectors express *E. coli*  $\beta$ -galactosidase from a CMV-driven minigene. Aliquots (5  $\mu$ g) of purified  $\beta$ -galactosidase from *E. coli* (Sigma) were resolved on a 10% SDS polyacrylamide gel (5 mg/lane) and electrophoretically transferred to a nitrocellulose  
30 membrane (Hybond-ECL, Amersham). The blot was incubated with blotto [5% nonfat milk, 50 mM Tris-HCl (pH 8.0), 2 mM CaCl<sub>2</sub>, and 0.05% Tween-20] at room temperature for 2 hours to block available sites. Individual lanes were cut and incubated with serum (diluted 1:200 in blotto)

for 1 hour at room temperature. Localization of antigen-antibody complex was accomplished by adding goat anti-mouse horseradish peroxidase conjugate, followed by ECL detection (Amersham). The cut lanes were reassembled on a mylar sheet prior to addition of ECL reagent and film documentation.

Intramuscular injection of the H5.010CMVlacZ E1 deleted adenovirus into skeletal muscle of C57BL/6 mice resulted in substantial  $\beta$ -galactosidase antibody accumulation in serum that did not occur in MHC-identical transgenic animals carrying an inserted lacZ gene which are immune tolerant to  $\beta$ -galactosidase. Significantly, neither C57BL/6 nor lacZ transgenic animals developed antibodies to  $\beta$ -galactosidase after intramuscular injection with AAV.CMVlacZ.

#### Example 5 - Comparative Studies of Adenoviral and AAV Vectors in Muscle Cells

Studies of the biology of muscle-directed gene transfer mediated by recombinant AAV and adenovirus demonstrate that adenoviruses, but not AAV, infect antigen presenting cells (APCs), which elicit a cascade of immunological responses leading to destructive cellular and humoral immunity.

An experimental paradigm was constructed to define the specific differences in host responses to skeletal muscle-directed gene transfer with recombinant AAV and adenovirus. The goal was to delineate differences in the biology of these vector systems that lead to preferential immunologic activation directed against a transgene product (i.e.,  $\beta$ -galactosidase) when expressed from a recombinant adenoviral vector, but not an AAV vector.

The general approach was to inject a lacZ expressing AAV into the right leg of a mouse. This has been shown

in the examples above to confer efficient and stable gene expression. In other experimental groups, the animals receive rAAV in addition to various combinations of vectors and cells in order to define components of the immune response directed against Ad that lead to destructive cellular and humoral immunity. The effects of these experimental manipulations were followed by assessing their impact on the stability of the rAAV engrafted muscle fibers, as well as measuring other immunologic parameters. Any intervention that elicits immunity to  $\beta$ -gal in muscle fibers can be detected by assessing the stability of transgene expression in the AAV-transduced muscle, and the development of inflammation.

Four experimental groups were developed for this study as summarized below. Virus was injected into mice using techniques substantially similar to those described in Example 2 above, i.e., virus was suspended in phosphate-buffered saline and injected directly into the tibialis anterior muscles. When the animals were necropsied, muscle tissues were snap-frozen in liquid nitrogen-cooled isopentane and sectioned at 6  $\mu$ m thickness, while serum samples and the draining inguinal lymph nodes were harvested for immunological assays.

Lymphocytes were harvested from inguinal lymph nodes and a standard 6 hr  $^{51}$ chromium (Cr)-release assay was performed essentially as described below, using different ratios of effector to target cells (C57SV, H-2<sup>b</sup>) in 200  $\mu$ l DMEM in V-bottom 96-well plates. Prior to mixing with the effector cells, target cells were either infected with an adenovirus expressing alkaline phosphatase (AdALP) or stably transduced with a lacZ-expressing retrovirus, pLJ-lacZ, labeled with 100  $\mu$ Ci of  $^{51}$ Cr and used at  $5 \times 10^3$  cells/well. After incubation for 6 hr,



aliquots of 100  $\mu$ l supernatant were counted in a gamma counter. The results for groups 1-3 are provided in Figs. 5A-5C.

5 Frozen sections (6 $\mu$ m) were fixed in methanol and stained with anti-CD4 and anti-CD8 antibodies. Morphometric analysis was performed to quantify the number of CD8+ cell and CD4+ cells per section.

10 The cytokine release assay was performed essentially as follows. Lymphocytes were restimulated for 40 hr with  $\beta$ -galactosidase, purified AAV, or adenovirus type 5. Cell-free supernatants (100  $\mu$ l) were assayed for the secretion of IL-10 and IFN- $\gamma$ . Proliferation was measured 72 hr later by a 8 hr  $^3$ H-thymidine (0.50  $\mu$ Ci/well) pulse. The results for the four groups are provided in Figs. 6A and 6B.

15 The neutralizing antibody assay was performed essentially as follows. Mouse serum samples were incubated at 56°C for 30 min to inactivate complement and then diluted in DMEM in two-fold steps starting from 20 1:20. Each serum dilution (100  $\mu$ l) was mixed with  $\beta$ -galactosidase or adenovirus type 5. After 60 min incubation at 37°C, 100  $\mu$ l of DMEM containing 20% FBS was added to each well. Cells were fixed and stained for  $\beta$ -galactosidase expression in the following day. All of 25 the cells stained blue in the absence of serum samples. The results for the four groups are provided in Figs. 6A and 6B.

30 Group 1 mice received AAV.CMVlacZ, produced as described in Example 1, in the right leg with no other intervention. Transduction with AAV.CMVlacZ alone led to high levels of stable gene transfer (evident even at 28 days) without infiltration of lymphocytes. No activation of CD8 T cells was detected (Fig. 5). Nor were antigen-specific, CD4<sup>+</sup> T cells [i.e., viral or  $\beta$ -galactosidase

(Figs. 6A and 6B)] detected. Antibodies were not generated to  $\beta$ -galactosidase or adenovirus (Figs. 7A and 7B).

Group 2 mice received AAV.CMVlacZ in the right leg and adenovirus expressing lacZ (H5.010CMVlacZ) in the left leg. The goal of this group was to determine if the immunologic response to the Ad-infected muscle fibers was systemic, as demonstrated by its impact on the biology of the contralateral AAV.CMVlacZ transduced leg.

10 Apparently, the adenoviral lacZ treatment induced an immune response to  $\beta$ -galactosidase that led to the destruction of the AAV lacZ transduced fibers. Not surprisingly, this was associated with infiltration of both CD4 and CD8 T cells into the AAV transduced leg, and

15 the activation of cytotoxic T lymphocytes to both adenoviral and  $\beta$ -galactosidase antigens (Fig. 8). Activated CD4 T cells specific for AAV, Ad, and  $\beta$ -gal antigens and antibodies specific for adenovirus and  $\beta$ -galactosidase were also observed.

20 Group 3 animals received a mixture of AAV.CMVlacZ and Ad BglIII in the right leg. AdBglIII is an E1-deleted adenovirus that expresses no recombinant gene. The goal of this group was to determine if the adenovirus provides an adjuvant effect which would elicit immunity to AAV to

25 lacZ in this setting. This did not lead to loss of transgene expression, although there was substantial infiltration of CD8 T cells and some activation of CD4 T cells to viral antigens, but not to  $\beta$ -galactosidase (Figs. 6A-6B). As expected, antibodies were generated

30 towards adenovirus but not towards  $\beta$ -galactosidase (Figs. 7A-7B).

Group 4 animals received AAV.CMVlacZ in the right leg and were adoptively transferred with antigen presenting cells harvested from naive animals and

35 infected ex vivo with adenovirus.

These animals mounted a vigorous and effective immunologic response to  $\beta$ -gal, as demonstrated by the loss of transgene expression, and the massive infiltration of CD8 and CD4 T cells. CD4 T cells were activated to  $\beta$ -gal in this experiment as shown in Figs. 6A-6B, and anti- $\beta$ -galactosidase antibodies generated, as shown in Figs. 7A-7B.

Example 6 - Transduction of a purified rAAV vector containing Factor IX into skeletal muscle cells and expression of F.IX at therapeutically useful levels and without eliciting a cytotoxic immune response.

The data provided in this example demonstrates that the method of this invention provides prolonged expression of a therapeutic transgene, F.IX, in both immunocompetent and immunoincompetent subjects in the absence of an immune response cytotoxic to the transduced cells. Further, the protein levels achieved in the serum of immunoincompetent animals are adequate to achieve a therapeutic effect. Thus, prolonged expression of human F.IX in muscle cells via rAAV vectors in immunoincompetent patients, such as those with hemophilia B, is useful to deliver F.IX to patients with that disease.

A. Preparation of purified rAAV

The rAAV vector used in the following in vivo experiments carries an expression cassette containing the human F.IX cDNA including a portion of Intron I under transcriptional control of the cytomegalovirus (CMV) immediate early gene promoter/enhancer and SV40 transcription termination signal. The vector, which contains this expression cassette flanked by AAV ITR sequences, and which completely lacks AAV protein coding sequences, was constructed as follows.

Recombinant AAV was generated by co-transfection of a F.IX *cis* plasmid (pAAV-FIX) and the trans-acting plasmid pAAV/Ad [A.W. Skulimowski and R.J. Samulski, Method. Mol. Genet., 7:7-12 (1995)] into human embryonic  
5 kidney (293) cells infected with an E1-deleted adenovirus as described by Fisher et al., J. Virol., 70:520-532 (1996). pAAV-FIX was derived from psub201 [Skulimowski and Samulski cited above] and contains the CMV promoter/enhancer, the human F.IX coding sequence  
10 including 1.4-kb fragment of Intron I [S. Kurachi et al, J. Biol. Chem., 270:5276-5281 (1995)], and the SV40 polyadenylation signal, flanked by AAV ITR sequences. The AAV *rep* and *cap* gene functions were supplied in trans by pAAV/Ad. The E1-deleted adenovirus contained a  $\beta$ -  
15 galactosidase (LacZ) or alkaline phosphatase (ALP) reporter gene to trace potential contamination of rAAV stocks with this helper virus. Cells were lysed 48 hours after transfection by sonication, and the released rAAV particles were purified by four rounds of CsCl density  
20 gradient centrifugation as described by Fisher et al., cited above.

The resulting rAAV-F.IX particles had a density of 1.37-1.40 g/ml. The titer of the purified rAAV-F.IX was determined by slot blot hybridization using a probe  
25 specific to either the CMV promoter or Intron I sequences and standards of pAAV-F.IX plasmid DNA of known concentration. The ability of rAAV-F.IX to transduce cells *in vitro* was confirmed by transducing growing HeLa cells and measuring the concentration of hF.IX in the  
30 culture supernatant 36 hours post-infection with an ELISA specific to hF.IX [J. Walter et al, Proc. Natl. Acad. Sci. USA, 93:3056-3061 (1996)]. rAAV-F.IX ( $10^{12}$ - $10^{13}$  genomes/ml) was stored at -79°C in HEPES-Buffered Saline, pH 7.8, including 5% glycerol.

Purified rAAV-F.IX routinely lacked detectable amounts of contaminating adenovirus when analyzed by transduction of 293 cells followed by staining for alkaline phosphatase or  $\beta$ -galactosidase as described by Fisher et al., cited above. Wild-type AAV was detected at  $<1$  infectious unit per  $10^9$  genomes of rAAV-F.IX. The assay for wild-type AAV was as follows: 293 cells grown on chamber slides were co-infected with adenovirus and with aliquots of purified rAAV-F.IX and fixed for immunofluorescence staining 24 hours post-infection. A mouse monoclonal antibody against AAV capsid proteins (American Research Products, Belmont, MA) served as a primary antibody, and anti-mouse IgG (DAKO Corporation, Carpinteria, CA) in a dilution of 1:40 as secondary antibody.

B. Introduction of rAAV into skeletal muscle

Mouse strains selected for intramuscular injection with rAAV were C57BL/6 (Charles River Laboratories, Wilmington, MA) and B6, 129, Rag 1 (Jackson Laboratories, Bar Harbor, Maine). Female mice (4-6 week-old) were anesthetized with an intraperitoneal injection of ketamine (70 mg/kg) and xylazine (10 mg/kg), and a 1 cm longitudinal incision was made in the lower extremity. AAV-F.IX ( $2 \times 10^{11}$  or  $1 \times 10^{10}$  vector genomes/animal in HEPES-buffered saline, pH 7.8) was injected into the tibialis anterior (25  $\mu$ l) and the quadriceps muscle (50  $\mu$ l) of each leg using a Hamilton syringe. Incisions were closed with 4-0 Vicryl suture. Blood samples were collected at seven-day intervals from the retro-orbital plexus in microhematocrit capillary tubes and plasma assayed for hF.IX by ELISA (part C below). For immunofluorescence staining (part D below) and DNA analysis (part F below), animals were sacrificed at selected time points and injected and non-injected muscle tissue was excised. Tissue was placed in OTC embedding

compound, snap-frozen in liquid nitrogen-cooled isopentane for seven seconds, and immediately transferred to liquid nitrogen.

C. Detection of human F.IX by ELISA

5 Human F.IX antigen in mouse plasma was determined by ELISA, as described by Walter et al., cited above. This ELISA did not cross-react with mouse F.IX. All samples were measured in duplicate. Protein extracts from injected mouse muscle were prepared by maceration of  
10 muscle in phosphate buffered saline (PBS) containing leupeptin (0.5 mg/ml) followed by sonication. Cell debris was removed by microcentrifugation, and 1:10 dilutions of the protein extracts were assayed for hF.IX by ELISA. Extracts from rAV.CMVLacZ (see Example 1  
15 above)-injected muscle were used as negative controls. Protein concentrations were determined with the BIORAD assay (Bio-Rad, Hercules, CA).

D. Immunofluorescence staining

In order to perform immunofluorescence staining  
20 of tissue sections, cryosections of muscle tissue (6 $\mu$ m) were fixed for 15 minutes in 3% paraformaldehyde in PBS, pH 7.4, rinsed in PBS for 5 minutes, incubated in methanol for 10 minutes, washed three times in PBS, and then blocked in PBS/3% bovine serum albumin (BSA) for 1  
25 hour. Sections were subsequently incubated overnight with an affinity purified goat anti-human F.IX antibody (Affinity Biologicals) that was diluted 1:1000 in PBS/1% BSA. After three washes (10 minutes each) in PBS/1% BSA, the secondary antibody was applied for 90 minutes (FITC-  
30 conjugated rabbit anti-goat IgG, DAKO Corporation, diluted 1:200 in PBS/1% BSA). After three additional washes in PBS/1% BSA, sections were rinsed in distilled water, air-dried and mounted with Fluoromount G mounting media (Fisher Scientific). All incubation steps were at  
35 room temperature, except for incubation with the primary

antibody (4°C). The same protocol was applied when sections were stained with rabbit anti-human collagen IV as primary antibody (Chemicon, Temecula, CA) in a 1:500 dilution and FITC conjugated anti-rabbit IgG (DAKO Corporation) as secondary antibody. For co-localization studies, a goat anti-hF.IX antibody conjugated to FITC (Affinity Biologicals) was applied simultaneously with the anti-collagen IV antibody, and rhodamine-conjugate anti-rabbit IgG (Chemicon) was used to detect collagen IV-antibody complexes. Fluorescence microscopy was performed with a Nikon FXA microscope.

E. Tests for circulating antibody against hF.IX

Plasma samples of C57BL/6 mice intramuscularly injected with AAV-F.IX were tested for the presence of antibodies against hF.IX using the ELISA. Microtiter plates were coated with human F.IX (1 µg/ml in 0.1M NaHCO<sub>3</sub>, pH 9.2). Dilute plasma samples (1:16) were applied in duplicate, and antibodies against hF.IX detected with horseradish peroxidase conjugated anti-mouse IgG (Zymed, San Francisco, CA) in a dilution of 1:2000. Buffer conditions were as described in Walter et al, cited above. Anti-hF.IX levels were estimated by comparison of absorbance values with monoclonal mouse anti-hF.IX (Boehringer Mannheim) diluted to a final concentration of 1 µg/ml. Western blots to demonstrate the presence of anti-hF.IX were performed as outlined by Dai et al., Proc. Natl. Acad. Sci. USA, 92:1401-1405 (1995), except that a horseradish peroxidase conjugated goat anti-mouse IgG antibody (Boehringer Mannheim) was used as secondary antibody, thereby allowing the detection of hF.IX-antibody complexes with ECL reagent (Amersham). Dilution of mouse plasma were 1:500.

F. DNA analyses

Genomic DNA was isolated from injected muscle tissue as described for mammalian tissue by Sambrook et

al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989). As described in Example 3 of the application, PCR reactions were carried out in order to amplify head-to-tail junctions of rAAV tandem repeats. The forward primer 005 [SEQ ID NO:4] anneals the SV40 polyadenylation signal (bp position 8014-8033), and reverse primers 013 [SEQ ID NO: 2] and 017 [SEQ ID NO: 3] bind to the CMV promoter (bp position 4625-4606 and 4828-4809). PCR reactions were performed using 100 ng genomic DNA in a total reaction volume of 100  $\mu$ l including 1.5 mM  $MgCl_2$ , and 0.5  $\mu$ M of primer pair 005/013 or 005/017. After an initial denaturation step (94°C for four minutes), 35 cycles of the following profile were carried out: denaturation at 94°C for four minutes), 35 cycles of the following profile were carried out: denaturation at 94°C for 1 minutes, annealing at 52°C for 1 minute, extension at 72°C for 90 seconds (10 minutes during the final cycle). PCR products were cloned (for DNA sequence analysis) using the T/A cloning kit (Invitrogen, San Diego, CA). Southern blot hybridizations were performed using  $^{32}P$ -dCTP random primed labelled probes specific for the CMV promoter (for hybridization to PCR fragments) or for intron I of hF.IX as present in rAAV-F.IX (for hybridization to genomic mouse DNA).

G. Results of expression of hF.IX in immunocompetent mice

Immunocompetent C57BL/6 mice were injected intramuscularly with rAAV-hF.IX and the animals sacrificed one month post-injection. An ELISA on protein extracts from injected muscles (tibialis anterior and quadriceps) demonstrated the presence of 1.8-2.1 ng hF.IX/mg tissue (40-50 ng hF.IX/mg protein). The expression of hF.IX in muscle tissues was confirmed by immunofluorescence studies on tissue sections.



Immunocompetent C57BL/6 mice were injected intramuscularly with rAAV-hF.IX and the animals sacrificed three months post-injection. Factor IX was not detected in uninjected muscle. Factor IX was not  
5 detected in muscle injected with a control, rAAV-lacZ. Expression of human F.IX was detected in muscle fibers of C57BL/6 mice three months post-injection ( $3.3 \times 10^{10}$  vector genomes per injection site; magnification 200x). Note  
10 that F.IX is present not only in the muscle fibers themselves, but in the interstitial spaces between the fibers as well where it appears to accumulate.

Interestingly, this staining pattern was identical to that seen with a polyclonal antibody against human collagen IV, which also stained the interstitial  
15 space. A stain of muscle one month post infection with rAAV-hF.IX ( $3.3 \times 10^{10}$  genomes per injection site) stained well with antibody to human collagen IV (data not shown). Collagen IV has recently been identified as a  
binding protein for human F.IX [W.-F. Cheung et al, Proc. Natl. Acad. Sci. USA, 93:11068-11073 (1996)].  
20

Initial experiments in immunocompetent mice demonstrated that, despite high levels of gene transfer and stable expression of hF.IX in injected muscle, it was not possible to detect significant amounts of hF.IX in  
25 the circulation by ELISA. See, Fig. 8. Further experiments demonstrated that the animals had developed high-titer antibodies against the circulating foreign protein. For example, when the same plasma samples were  
tested for antibodies against human F.IX, a strong  
30 antibody response was seen in all injected animals starting at day 11 post-injection. See Fig. 9. Using Western blot analysis, high levels of circulating antibody were found to have persisted for the duration of the experiment.

This finding contrasted with our previously reported experience, in which a different route of administration, i.e., intravenous injection, of a different vector, i.e., an adenoviral vector expressing hF.IX, effected a different immune response, i.e., did not trigger formation of neutralizing antibodies against hF.IX (Walter et al, cited above)].

Levels of protein expression required to induce antibody formation however are quite low. The Western blot assay is somewhat less sensitive than an ELISA but documents what appears to be a rising antibody titer beginning 18 days after injection. Thus in the immunocompetent animals, the absence of detectable hF.IX expression at initial time points is a result of the biology of rAAV expression in muscle, whereas the subsequent absence of detectable F.IX in the circulation results from the production of antibodies to the foreign protein. Nevertheless the serum antibody response was not associated with cytotoxic immune response directed against the transgene-expressing cells. In fact, neither inflammation nor extensive tissue damage was observed in any of the tissue sections discussed above nor in sections analyzed by H&E staining (data not shown). This contrasts with the immune response elicited by injection of skeletal muscle with recombinant adenovirus carrying a transgene [Dai et al, cited above; Y. Yang et al, Hum. Molec. Genet., 5:1703-1712 (1996); X. Xiao et al, J. Virol., 70:8098-8108 (1996)].

H. The expression of hF.IX in immunodeficient mice

AAV-F.IX was delivered to muscles of Rag 1 mice, which are homozygous for a mutation in the recombinase activating gene 1. These animals are therefore functionally equivalent to severe combined immunodeficiency (SCID) mice and do not produce mature B or T cells. A dose of  $2 \times 10^{11}$  rAAV-hF.IX vector genomes

per animal resulted in stable expression of hF.IX in mouse plasma. See Fig. 10. Human F.IX was first detectable by ELISA in the second week after the injection and rose gradually thereafter. Plasma levels in all animals reached a plateau of therapeutic levels of F.IX five to seven weeks post-injection at 200 to 350 ng hF.IX/ml mouse plasma. This level was maintained for the duration of the experiment (four months post-injection). When a total of  $1 \times 10^{10}$  rAAV-hF.IX vector genomes was injected, expression was three- to four-fold lower, but still reached therapeutic levels ( $>100$  ng/ml) for some animals. See, Fig. 11. These levels, which represent 4-7% of normal circulating levels in plasma, are well within a therapeutic range, and demonstrate that the method of this invention is a feasible for the treatment of hemophilia, a disease associated with immunodeficiency.

#### I. Results of DNA Analysis

Genomic DNA from injected muscle tissue was isolated six to eight weeks post-injection. The presence of introduced vector DNA was demonstrated by digestion with EcoRV, which releases a 1.8-kb fragment from the vector construct including the entire 1.4-kb intron I sequence. A probe specific to intron I hybridized to this fragment and did not cross-hybridize to mouse DNA from an uninjected animal. Undigested DNA showed as hybridization signal in the high molecular weight DNA. Furthermore, PCR primers designed to amplify junction sequences of head-to-tail concatamers of recombinant AAV present in transduced cells (Fig. 12) successfully amplified such sequences from muscle DNA isolated from AAV-F.IX transduced tissue (tibialis anterior and quadriceps of immunodeficient and immunocompetent animals). The PCR products were visualized by Southern blot hybridization with a probe specific to the CMV

promoter/enhancer. Primer pair 005-013 produced fragments that were 1.0-kb and smaller; primer pair 005-017 amplified fragments that were 1.2-kb and smaller. As expected, these PCR reactions resulted not in distinct  
5 bands of the sizes noted above, but rather in a series of amplification products with a maximum size as predicted, because of imprecise joining of AAV genomes present in these tandem repeats [S. K. McLaughlin et al, J. Virol.,  
10 62:1963-1973 (1988)]. The imprecise joining results from variable deletions of ITR sequences at the junction sites as confirmed by DNA sequencing of cloned PCR products (data not shown).

#### J. PCR studies

While head-to-head and tail-to-tail  
15 arrangements of AAV genomes can occur during viral replication [K. I. Berns, Microbiol. Rev., 54:316-329 (1990)], head-to-tail arrays are more typically associated with AAV that has been integrated into the chromosomal DNA of the transduced cell during latent  
20 infection [S. K. McLaughlin et al, J. Virol., 62:1963-1973 (1988); J. D. Tratschin et al, Mol. Cell Biol., 5:3251-3260 (1985); N. Muzcyka, Current Topics in Microbiology and Immunology, 158:97-129 (1992)].

Southern blot data on undigested rAAV-injected  
25 muscle cell DNA demonstrated that the rAAV DNA derived from host cell genomic DNA six weeks after injection is present as a high molecular weight species. The higher signal intensity seen with the restricted DNA likely results from an unmasking effect when the fragments are  
30 separated from the bulk of genomic DNA [X. Xiao et al, J. Virol., 70:8089-8108 (1996)]. This finding, the presence of a hybridization signal for high molecular weight DNA, is, like the PCR data, consistent with integrative events occurring during transduction. The integration state of

the rAAV discussed in this paragraph and in paragraph I is also likely to contribute to the stability and prolonged expression of the transgene.

5     Example 7 - Expression of ApoE using rAAV Administered to a Skeletal Muscle Cell

       The following example demonstrates the prolonged expression of another therapeutic transgene product, apolipoprotein E (ApoE), a protein useful in the treatment of atherosclerosis, by introduction into  
10    skeletal muscle of a rAAV vector according to this invention. Again, the absence of a destructive CTL response permits the prolonged expression of the transgene product.

       A.     Construction of the rAAV

15         Recombinant AAV vectors encoding the secreted protein human ApoE were constructed in a manner similar to that described above for F.IX. ApoE cDNA was excised from a plasmid pAlterApoE3 (contributed by Dr. Rader's laboratory, University of Pennsylvania) with XbaI  
20    digestion, blunted and cloned into NotI digested pCMVLacZ backbone (see the vector construction diagram of Fig. 13). A 2062 bp SmaI/SacI fragment from the lacZ gene in pCMVLacZ was isolated and inserted into the SalI site of the new plasmid as a stuffer. The ApoE minigene  
25    cassette, which now contained the CMV promoter, ApoE cDNA SV40 polyadenylation sequences and a 2062 bp stuffer, was isolated by EcoRI/HindIII digestion (total length: 4.3 kb) and then ligated to an XbaI digested pSub201 backbone. The final product, designated pSubCMVApoE-  
30    2062RO, was used as the cis plasmid in the production of rAAV.ApoE production following the procedures described above for rAAV.F.IX.

B. In vitro analysis of ApoE expression by rAAV.ApoE

84-31 cells seeded in 6 well plates were infected with 2 microliters of CsCl purified rAAV.ApoE in 2 ml Dulbeccos Modified Eagles Medium with 2% fetal bovine serum. The cells were kept at 37°C for 48 hours. An aliquot of the supernatant was then removed from the well for a Western blot analysis of ApoE. The results showed ApoE protein was clearly detectable in the supernatant of 84-31 cells infected with AAV.ApoE.

C. In vivo expression of rAAV.ApoE in ApoE knockout mice

2.5 to 5 X 10<sup>10</sup> particles of rAAV.ApoE were injected into anterior tibialis and quadriceps muscles on each side of ApoE knockout mice (total particles per mouse: 5 X 10<sup>10</sup> to 5 x 10<sup>11</sup>). Prolonged local ApoE expression was detectable by immunofluorescence at day 28 and day 120 post-injection even in the presence of plasma anti-ApoE antibodies.

D. Conclusion

This experiment demonstrates that intramuscular injection of the vector, rAAV-ApoE, into Apo-E knockout mice leads to muscle fiber transduction and secretion of substantial quantities of the recombinant protein in the circulation. Prolonged expression of the transgene in the muscle fiber in the absence of a destructive CTL immune response was achieved, even though humoral immunity was elicited to the secreted protein.

Example 8 - Transgene expression in a primate

A rhesus monkey was anesthetized, the forearm was clipped and aseptically prepared and a 0.5 cm incision was made in the skin over the tibialis anterior muscle. The fascia was identified and the rAV.CMVLacZ (described in Example 1) viral suspension (175 microliter of 10<sup>2</sup>

genomes/ml) was injected 5-7 mm deep into the fascia. Fourteen days later, a muscle biopsy was removed, frozen in OCT, sectioned and stained in X-gal. Tissue sections were quantitatively analyzed using the Leica Z500MC Image Processing and Analysis System interfaced with a Nikon FXA Microscope. X-gal histochemistry revealed high level  $\beta$ -galactosidase expression in the majority of muscle fibers in the area of the injection site. Twenty percent of fibers expressed  $\beta$ -galactosidase in a 224 mm<sup>2</sup> area in the region of the injections. Based on the above-described results with ApoE and F.IX, expression is expected to be prolonged in the absence of a cytotoxic immune response. These data support that the expression of a transgene according to this invention can be duplicated in an animal other than a mouse, and particularly in a primate animal.

Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the processes of the present invention are believed to be encompassed in the scope of the claims appended hereto.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Trustees of the University of Pennsylvania  
Wilson, James M.  
Fisher, Krishna J.
- (ii) TITLE OF INVENTION: Method for Recombinant Adeno-Associated  
Virus-Directed Gene Therapy
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
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  - (E) COUNTRY: USA
  - (F) ZIP: 19477
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: WO
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 08/708,188
  - (B) FILING DATE: 06-SEP-1996
- (viii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 08/729,061
  - (B) FILING DATE: 10-OCT-1996
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Kodroff, Cathy A.
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- (ix) TELECOMMUNICATION INFORMATION:
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  - (B) TELEFAX: 215-540-5818

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 10398 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: unknown
- (ii) MOLECULE TYPE: cDNA



## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAATTCGCTA GCATCATCAA TAATATACCT TATTTTGGAT TGAAGCCAAT ATGATAATGA	60
GGGGGTGGAG TTTGTGACGT GGCGCGGGGC GTGGGAACGG GGCGGGTGAC GTAGTAGTGT	120
GGCGGAAGTG TGATGTTGCA AGTGTGGCGG AACACATGTA AGCGACGGAT GTGGCAAAAG	180
TGACGTTTTT GGTGTGCGCC GGTGTACACA GGAAGTGACA ATTTTCGCGC GGTTTTAGGC	240
GGATGTTGTA GTAAATTG GCGTAACCGA GTAAGATTG GCCATTTTCG CGGGAAACT	300
GAATAAGAGG AAGTGAAATC TGAATAATTT TGTGTTACTC ATAGCGCGTA ATATTTGTCT	360
AGGGAGATCT GCTGCGCGCT CGCTCGCTCA CTGAGGCCGC CCGGGCAAAG CCCGGGCGTC	420
GGGCGACCTT TGGTCGCCCC GCCTCAGTGA GCGAGCGAGC GCGCAGAGAG GGAGTGGCCA	480
ACTCCATCAC TAGGGGTTCC TTGTAGTTAA TGATTAACCC GCCATGCTAC TTATCTACAA	540
TTGAGCTTG CATGCCTGCA GGTCGTTACA TAACTTACGG TAAATGGCCC GCCTGGCTGA	600
CCGCCCAACG ACCCCCGCCC ATTGACGTCA ATAATGACGT ATGTTCCCAT AGTAACGCCA	660
ATAGGGACTT TCCATTGACG TCAATGGGTG GAGTATTTAC GGTAAACTGC CCACTTGGCA	720
GTACATCAAG TGTATCATAT GCCAAGTACG CCCCTATTG ACGTCAATGA CGGTAAATGG	780
CCCGCCTGGC ATTATGCCCA GTACATGACC TTATGGGACT TTCCTACTTG GCAGTACATC	840
TACGTATTAG TCATCGCTAT TACCATGGTG ATGCGGTTTT GGCAGTACAT CAATGGGCGT	900
GGATAGCGGT TTGACTCAGG GGGATTTCCA AGTCTCCACC CCATTGACGT CAATGGGAGT	960
TTGTTTTGGC ACCAAAATCA ACGGGACTTT CCAAATGTC GTAACAACCTC CGCCCCATTG	1020
ACGCAAATGG GCGGTAGGCG TGTACGGTGG GAGGTCTATA TAAGCAGAGC TCGTTTAGTG	1080
AACCGTCAGA TCGCCTGGAG ACGCCATCCA CGCTGTTTTG ACCTCCATAG AAGACACCGG	1140
GACCGATCCA GCCTCCGGAC TCTAGAGGAT CCGGTACTCG AGGAACTGAA AAACCAGAAA	1200
GTTAACTGGT AAGTTTAGTC TTTTGTCTT TTATTTTCTG TCCCGGATCC GGTGGTGGTG	1260
CAAATCAAAG AACTGCTCCT CAGTGGATGT TGCCTTTACT TCTAGGCCTG TACGGAAGTG	1320
TTACTTCTGC TCTAAAAGCT GCGGAATTGT ACCCGCGGCC GCAATTCCCG GGGATCGAAA	1380
GAGCCTGCTA AAGCAAAAAA GAAGTCACCA TGTCGTTTAC TTTGACCAAC AAGAACGTGA	1440
TTTTCGTTGC CGGTCTGGGA GGCATTGGTC TGGACACCAG CAAGGAGCTG CTCAAGCGCG	1500
ATCCCGTCGT TTTACAACGT CGTGAAGTGG AAAACCTGG CGTTACCCAA CTTAATCGCC	1560
TTGCAGCACA TCCCCCTTTC GCCAGCTGGC GTAATAGCGA AGAGGCCCGC ACCGATCGCC	1620
CTTCCCAACA GTTGCGCAGC CTGAATGGCG AATGGCGCTT TGCTGGTTT CCGGCACCAG	1680
AAGCGGTGCC GGAAAGCTGG CTGGAGTGCG ATCTTCTCTGA GGCCGATACT GTCGTCTGTC	1740
CCTCAAACCTG GCAGATGCAC GGTTACGATG CGCCCATCTA CACCAACGTA ACCTATCCCA	1800
TTACGGTCAA TCCGCCGTTT GTTCCACCGG AGAATCCGAC GGGTTGTTAC TCGCTCACAT	1860

TTAATGTTGA TGAAAGCTGG CTACAGGAAG GCCAGACGCG AATTATTTTT GATGGCGTTA	1920
ACTCGGCGTT TCATCTGTGG TGCAACGGGC GCTGGGTCGG TTACGGCCAG GACAGTCGTT	1980
TGCCGTCTGA ATTTGACCTG AGCGCATT TTACGCGCCG AGAAAACCGC CTCGCGGTGA	2040
TGGTGCTGCG TTGGAGTGAC GGCAGTTATC TGGAAGATCA GGATATGTGG CGGATGAGCG	2100
GCATTTTCCG TGACGTCTCG TTGCTGCATA AACCGACTAC ACAAATCAGC GATTTCATG	2160
TTGCCACTCG CTTTAATGAT GATTTTCAGCC GCGCTGTACT GGAGGCTGAA GTTCAGATGT	2220
GCGGCGAGTT GCGTGACTAC CTACGGGTAA CAGTTTCTTT ATGGCAGGGT GAAACGCAGG	2280
TCGCCAGCGG CACCGCGCCT TTCGGCGGTG AAATTATCGA TGAGCGTGGT GGTATGCCG	2340
ATCGCGTCAC ACTACGTCTG AACGTCGAAA ACCCGAAACT GTGGAGCGCC GAAATCCCGA	2400
ATCTCTATCG TGCGGTGGTT GAACTGCACA CCGCCGACGG CACGCTGATT GAAGCAGAAG	2460
CCTGCGATGT CGGTTTCCGC GAGGTGCGGA TTGAAAATGG TCTGCTGCTG CTGAACGGCA	2520
AGCCGTTGCT GATTTCGAGGC GTTAACCGTC ACGAGCATCA TCCTCTGCAT GGTTCAGTCA	2580
TGGATGAGCA GACGATGGTG CAGGATATCC TGCTGATGAA GCAGAACAAC TTTAACGCCG	2640
TGCGCTGTTT GCATTATCCG AACCATCCGC TGTGGTACAC GCTGTGCGAC CGCTACGGCC	2700
TGTATGTGGT GGATGAAGCC AATATTGAAA CCCACGGCAT GGTGCCAATG AATCGTCTGA	2760
CCGATGATCC GCGCTGGCTA CCGGCGATGA GCGAACCGGT AACCGGAATG GTGCAGCGCG	2820
ATCGTAATCA CCCGAGTGTG ATCATCTGGT CGCTGGGGAA TGAATCAGGC CACGGCGCTA	2880
ATCAGGACGC GCTGTATCGC TGGATCAAAT CTGTCGATCC TTCCCGCCCG GTGCAGTATG	2940
AAGGCGGCGG AGCCGACACC ACGGCCACCG ATATTATTG CCCGATGTAC GCGCGCGTGG	3000
ATGAAGACCA GCCCTTCCCG GCTGTGCCGA AATGGTCCAT CAAAAAATGG CTTTCGCTAC	3060
CTGGAGAGAC GCGCCCGCTG ATCCTTTGCG AATACGCCCA CGCGATGGGT AACAGTCTTG	3120
GCGGTTTCGC TAAATACTGG CAGGCGTTTC GTCAGTATCC CCGTTTACAG GGCGGCTTCG	3180
TCTGGGACTG GGTGGATCAG TCGCTGATTA AATATGATGA AAACGGCAAC CCGTGGTCGG	3240
CTTACGGCGG TGATTTTGGC GATACGCCGA ACGATCGCCA GTTCTGTATG AACGGTCTGG	3300
TCTTTGCCGA CCGCACGCCG CATCCAGCGC TGACGGAAGC AAAACACCAG CAGCAGTTTT	3360
TCCAGTTCCG TTTATCCGGG CAAACCATCG AAGTGACCAG CGAATACCTG TTCCGTCATA	3420
GCGATAACGA GCTCCTGCAC TGGATGGTGG CGCTGGATGG TAAGCCGCTG GCAAGCGGTG	3480
AAGTGCCTCT GGATGTCGCT CCACAAGGTA AACAGTTGAT TGAAGTGCCT GAAGTACCGC	3540
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52

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## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CATGGTAATA GCGATGACTA

20

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCTCTGCTTA TATAGACCTC

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53

## (2) INFORMATION FOR SEQ ID NO:4:

- (1) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATAAGCTGCA ATAAACAAGT

20

What is claimed is:

1. Use of a recombinant adeno-associated virus (rAAV) comprising a heterologous gene operably linked to sequences which control expression thereof in a cell for the manufacture of a medicament for reducing the immune response to the rAAV, wherein the rAAV is substantially free of contamination with a helper virus and is administered to a skeletal muscle cell.
2. Use of recombinant adeno-associated virus (rAAV) comprising a transgene operably linked to sequences which control expression thereof in a cell for the manufacture of a medicament for prolonging expression of the transgene, wherein the rAAV is substantially free of contamination with a helper virus and is administered to a skeletal muscle cell.
3. Use according to claim 1 or 2, wherein the transgene is a secretable protein.
4. Use according to claim 3, wherein the protein is selected from the group consisting of Factor IX, ApoE,  $\beta$ -interferon, insulin, erythropoietin, growth hormone, and parathyroid hormone.
5. Use according to any of claims 1 to 4, wherein the rAAV consists of, from 5' to 3', 5' AAV inverse terminal repeats (ITRs), a heterologous promoter, the transgene, a polyadenylation sequence, and 3' AAV ITRs.
6. Use according to claim 1 or 2, wherein the transgene is a dystrophin gene.



7. A method for expressing a transgene in a skeletal muscle cell in the absence of a cytotoxic immune response directed against the cell, comprising the step of introducing into the cell a recombinant adeno-associated virus (rAAV) comprising a transgene operably linked to sequences which control its expression, wherein the rAAV is substantially free of contamination with a helper virus and wherein the transgene is expressed in the cell.

8. The method according to claim 7, wherein the transgene is a secretable protein.

9. The method according to claim 8, wherein the protein is selected from the group consisting of Factor IX, ApoE,  $\beta$ -interferon, insulin, erythropoietin, growth hormone, and parathyroid hormone.

10. The method according to claim 7, wherein the rAAV consists of, from 5' to 3', 5' AAV inverse terminal repeats (ITRs), a heterologous promoter, the transgene, a polyadenylation sequence, and 3' AAV ITRs.

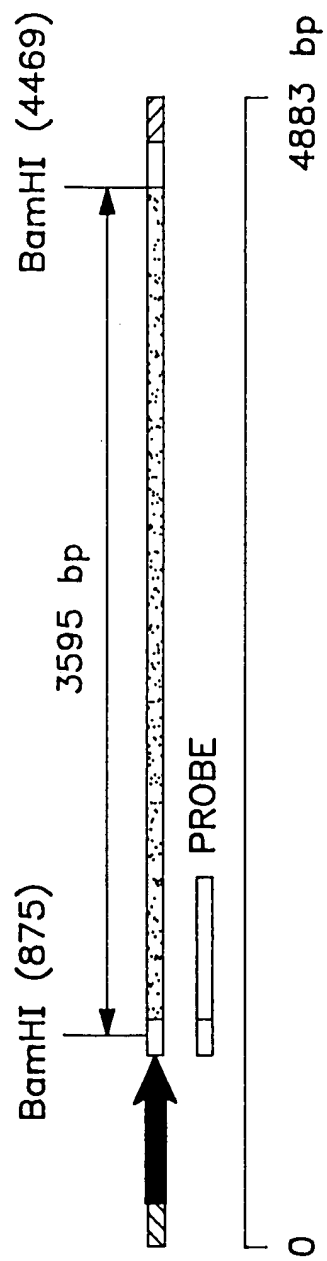


FIG. 1

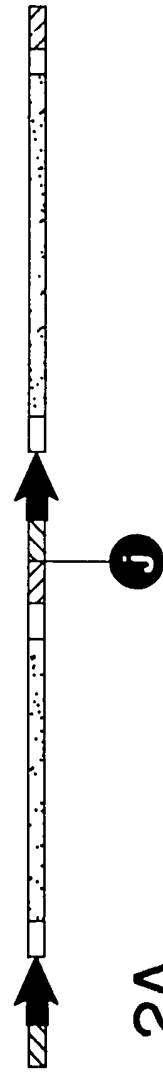


FIG. 2A

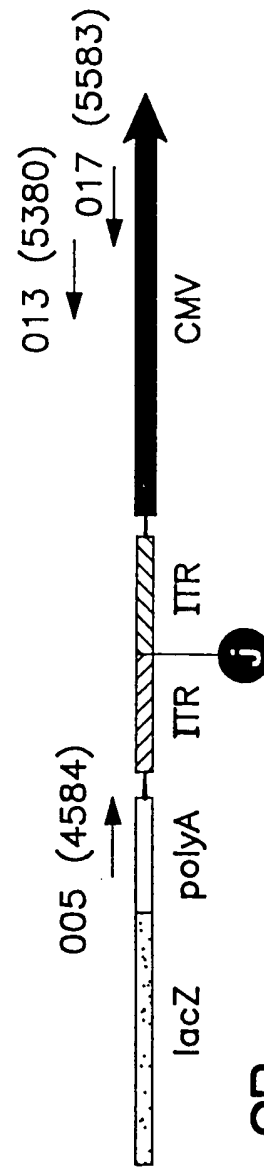


FIG. 2B

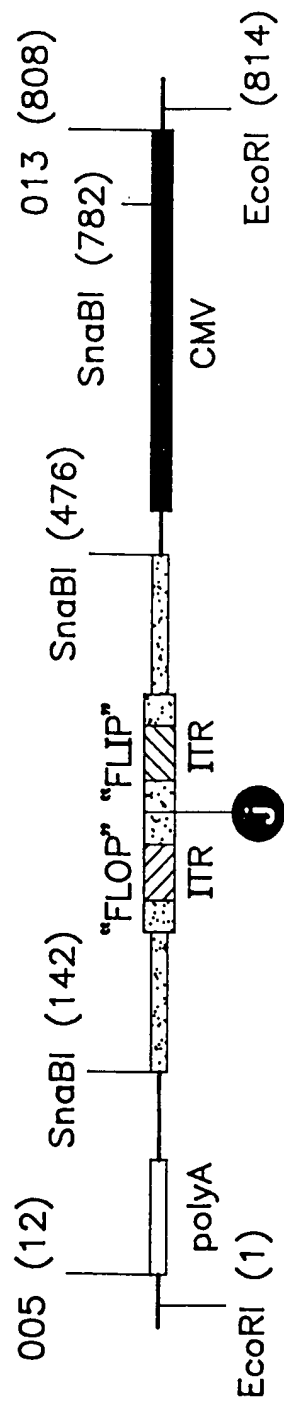


FIG. 3

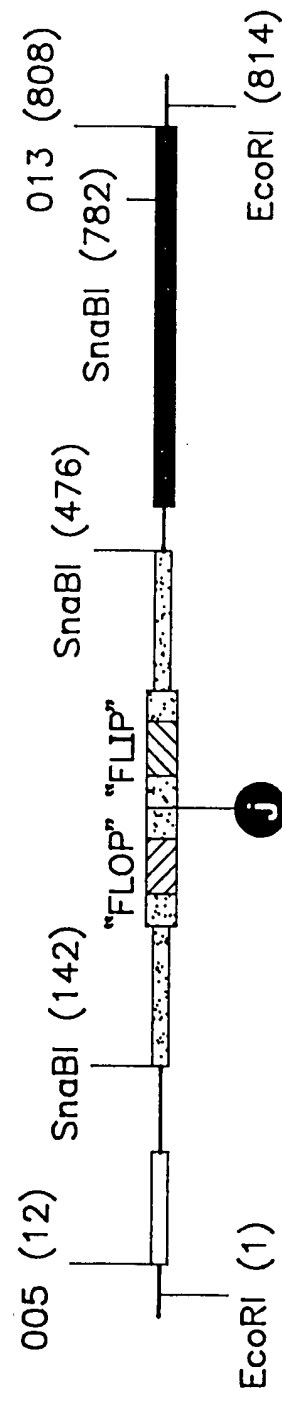


FIG. 4A

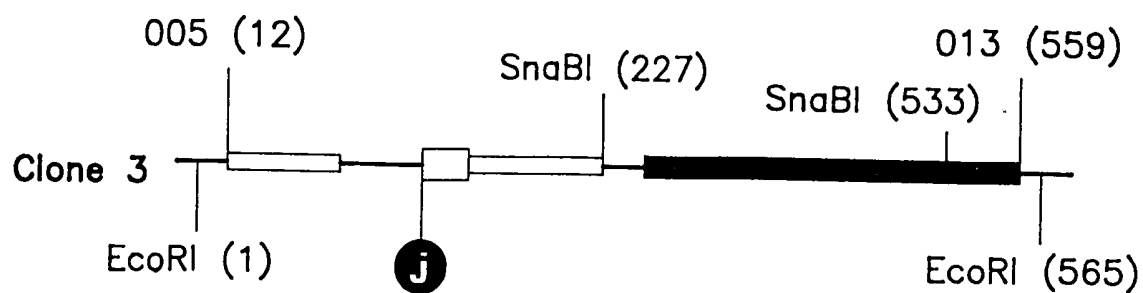


FIG. 4B

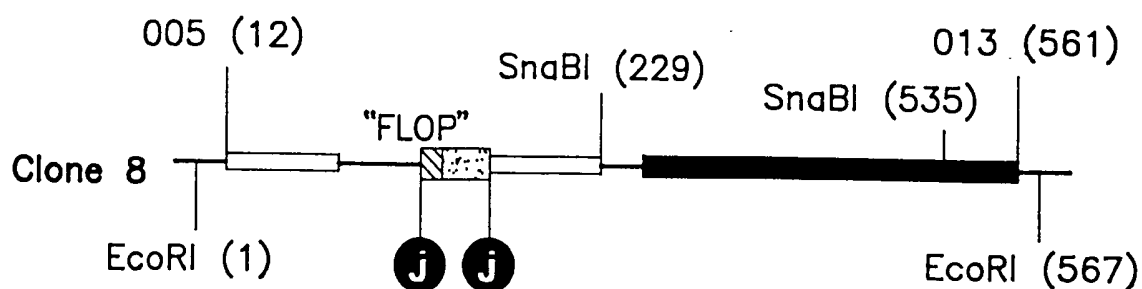


FIG. 4C

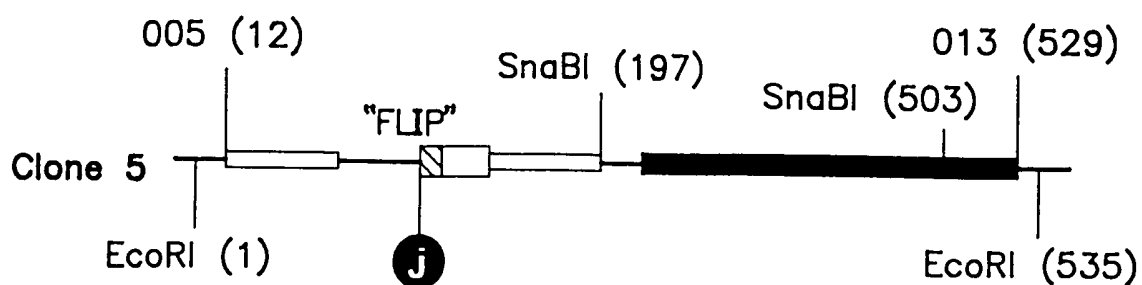


FIG. 4D

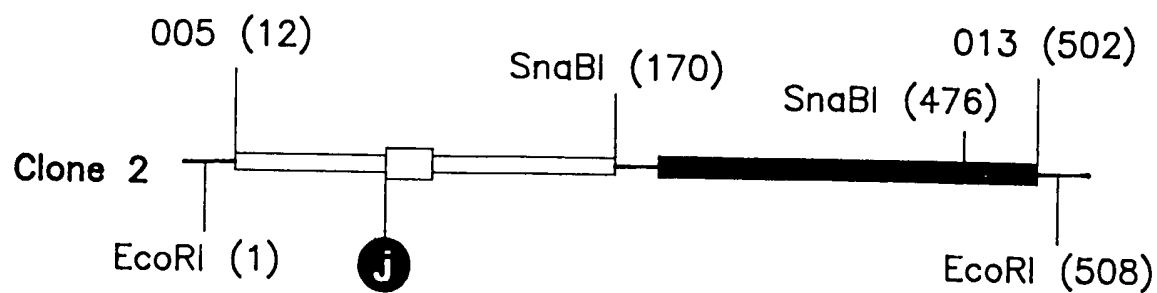


FIG. 4E

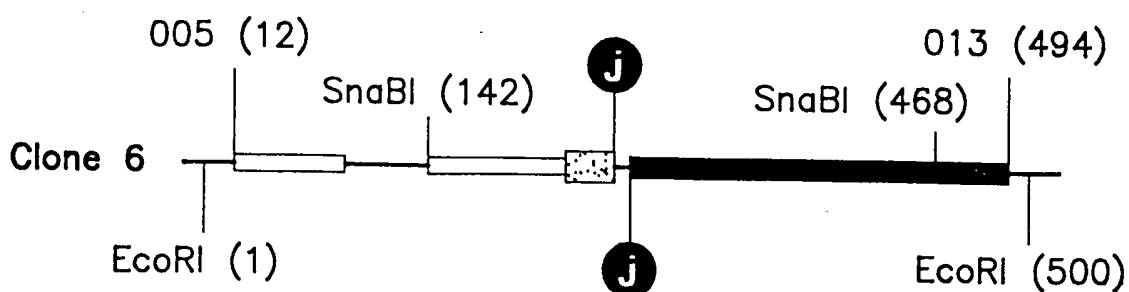


FIG. 4F

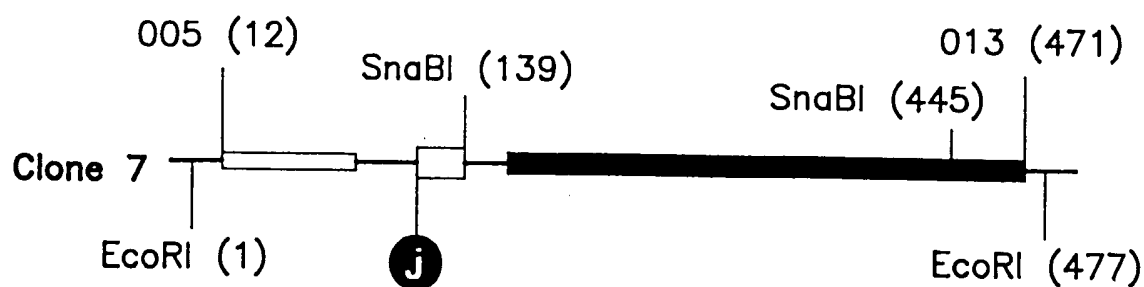


FIG. 4G

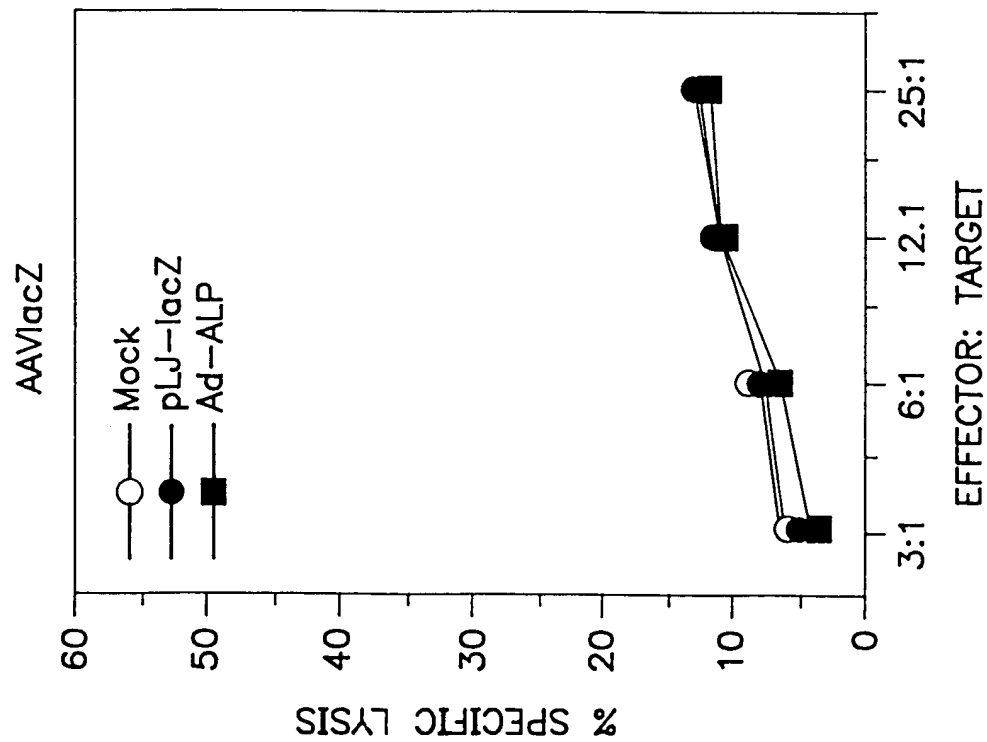


FIG. 5A

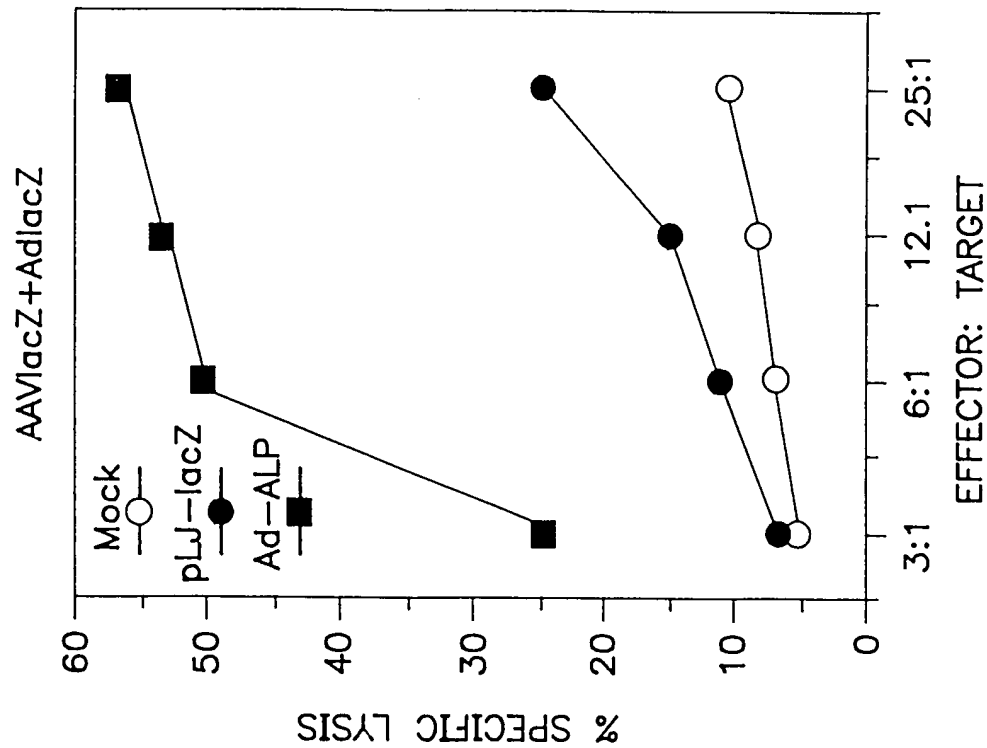


FIG. 5B



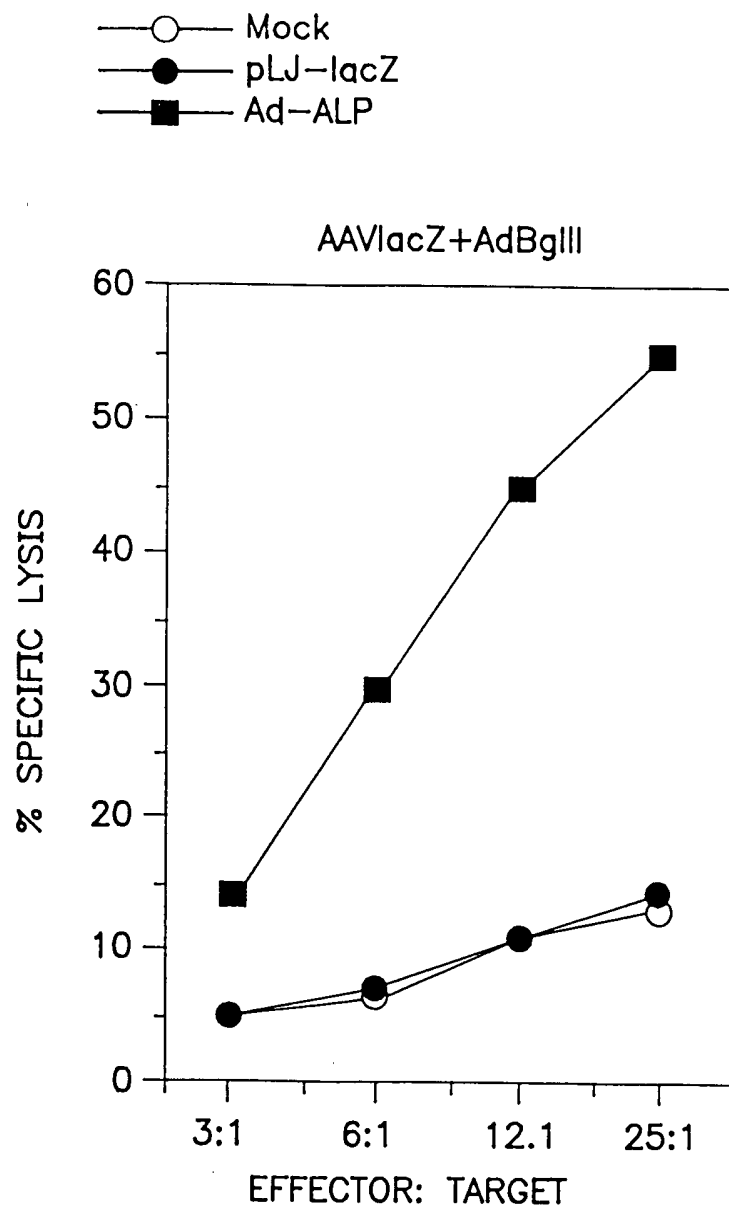


FIG. 5C

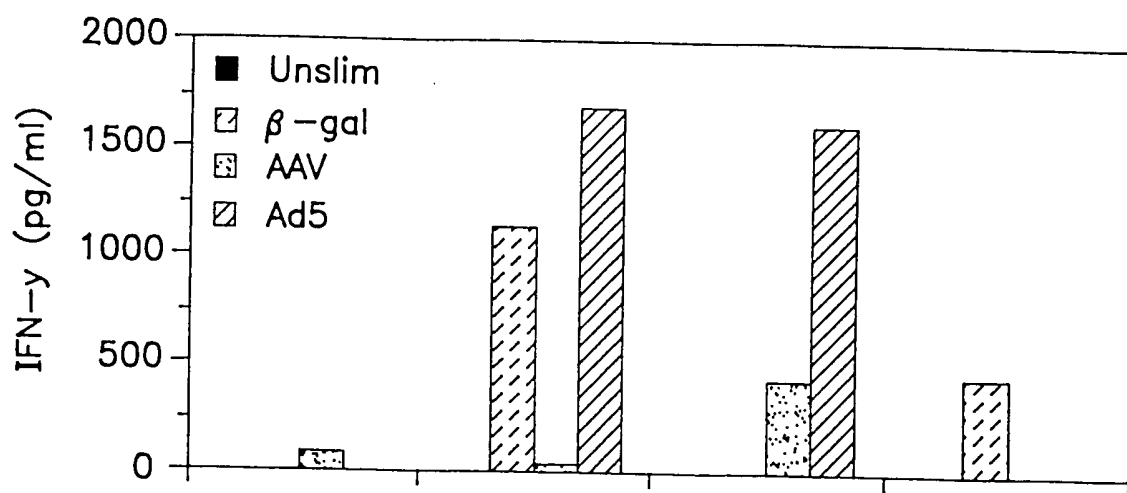


FIG. 6A

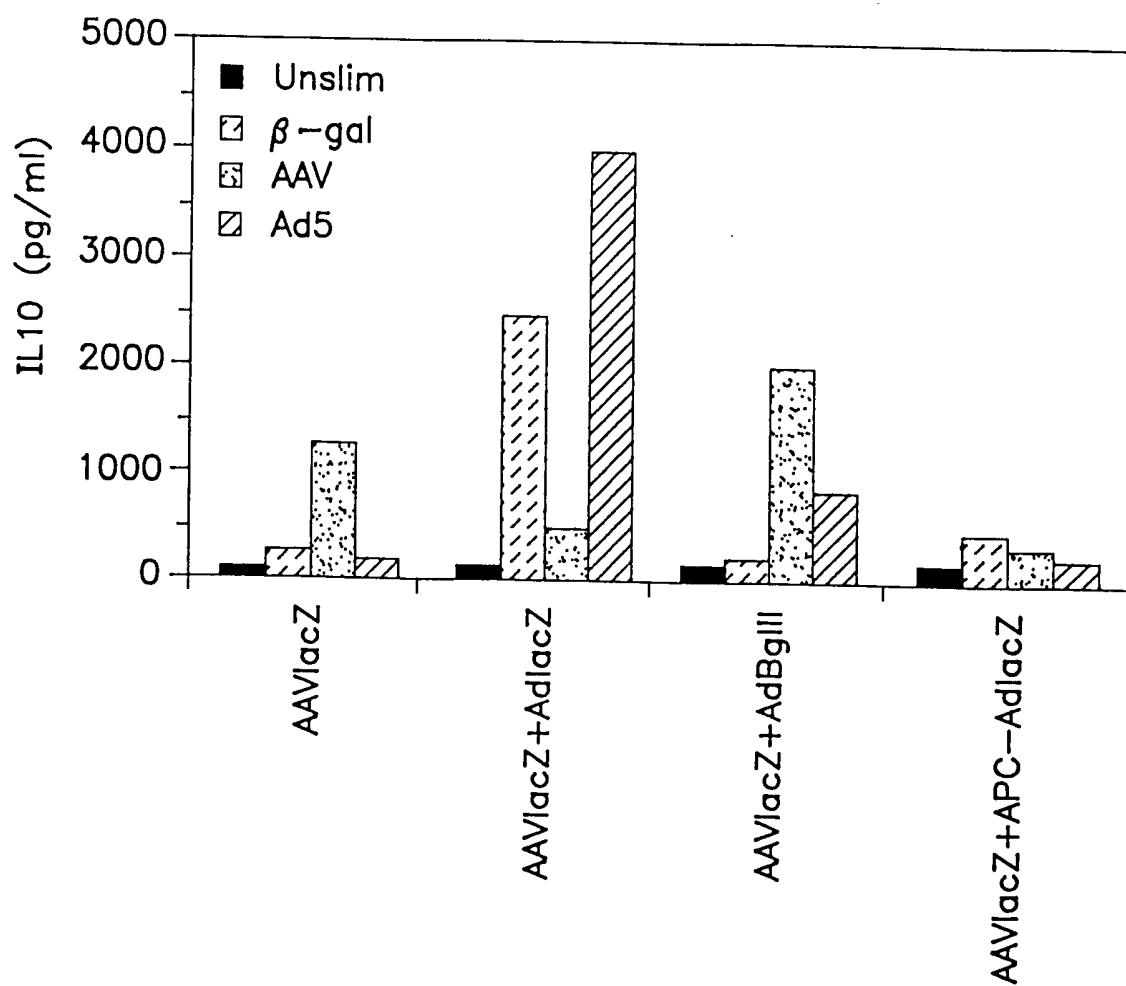


FIG. 6B

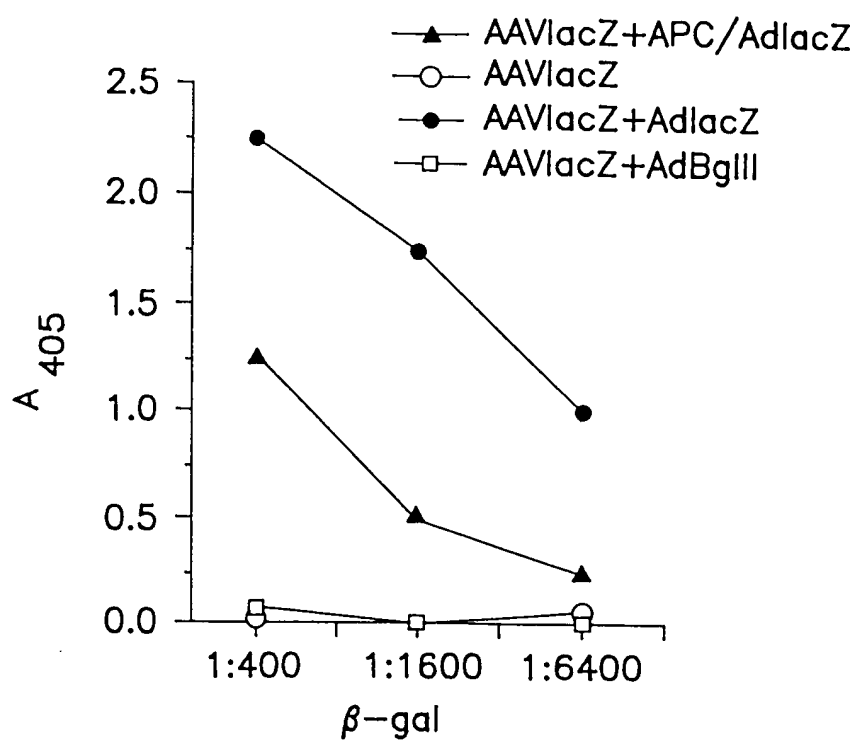


FIG. 7A

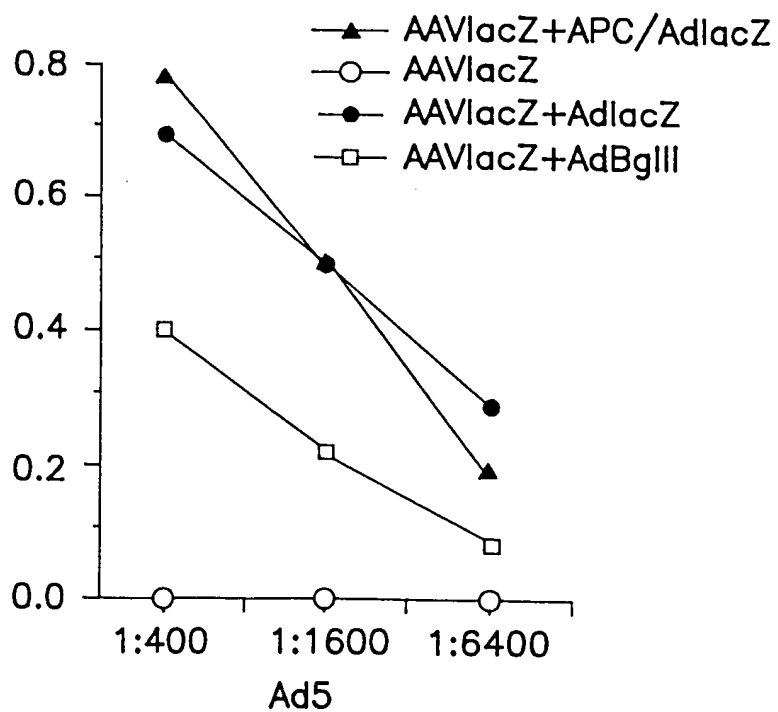


FIG. 7B

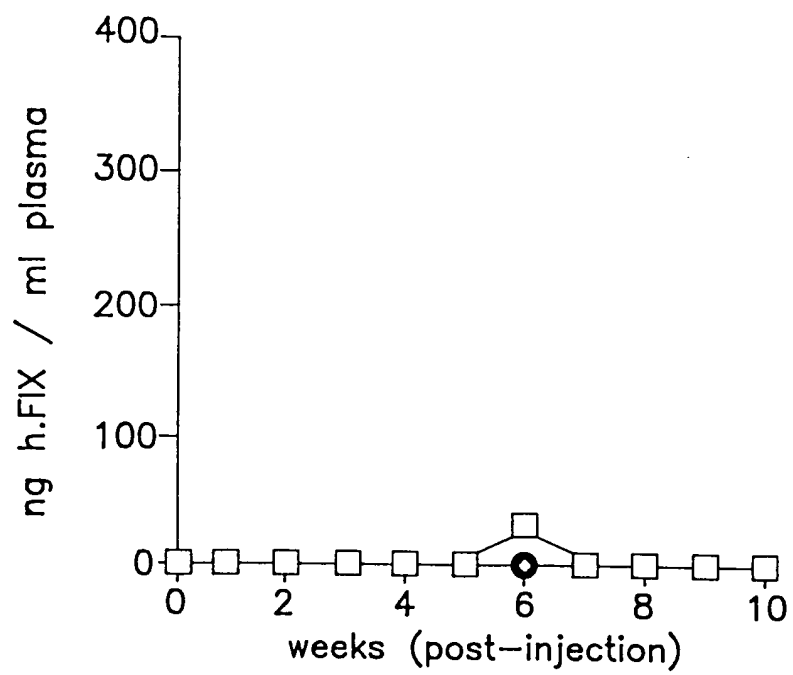


FIG. 8

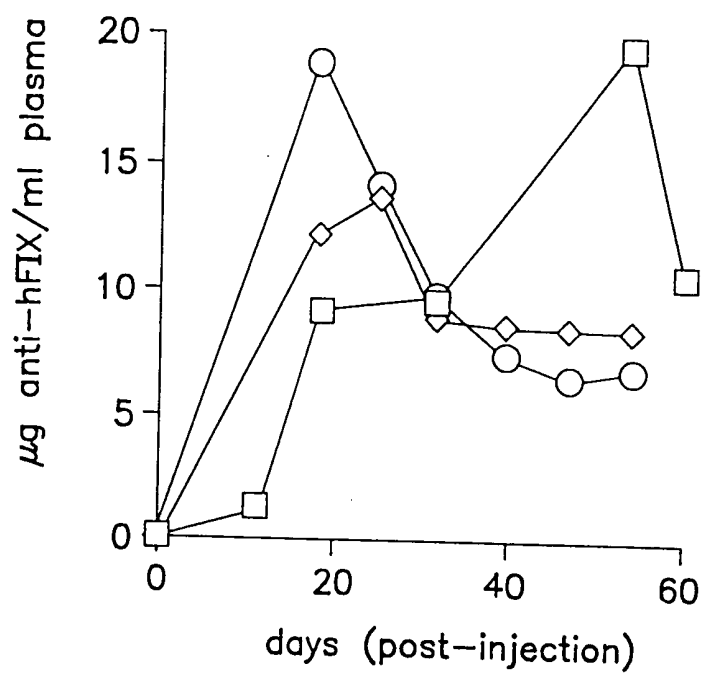


FIG. 9

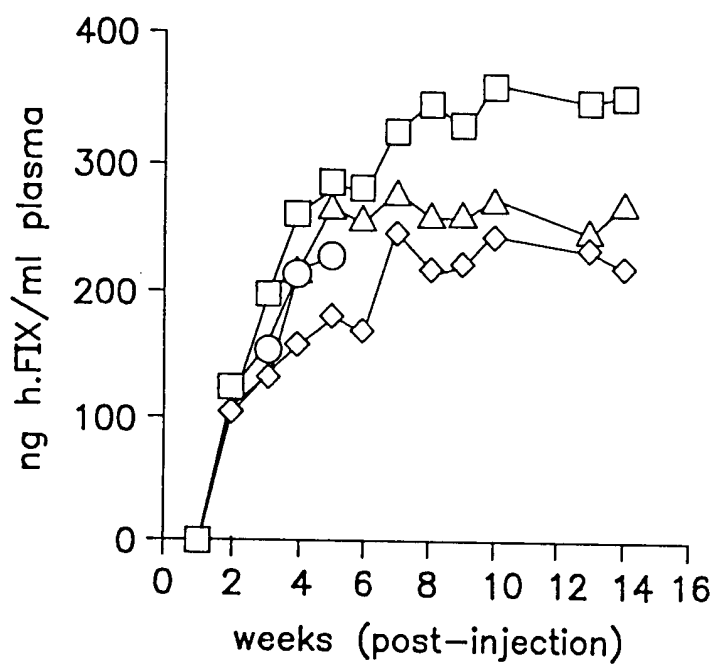


FIG. 10

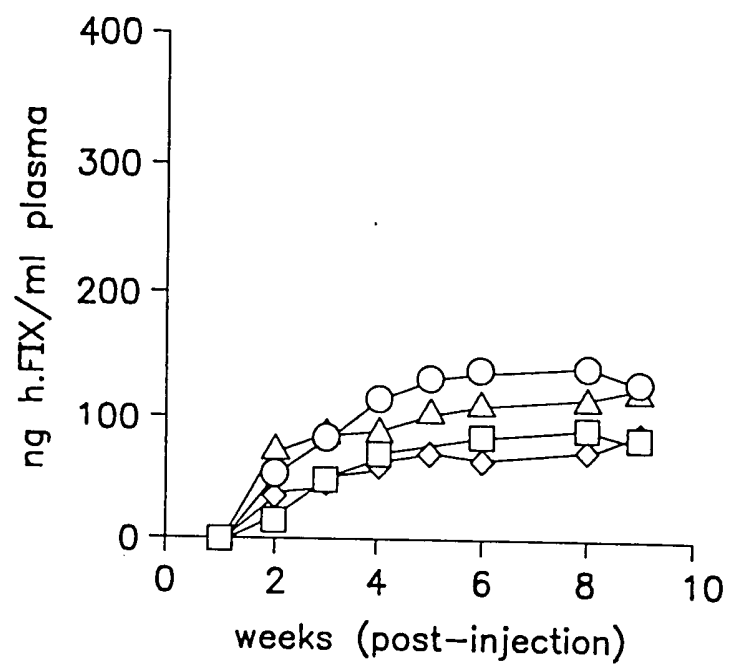


FIG. 11





# Construction of AV.CMV ApoE

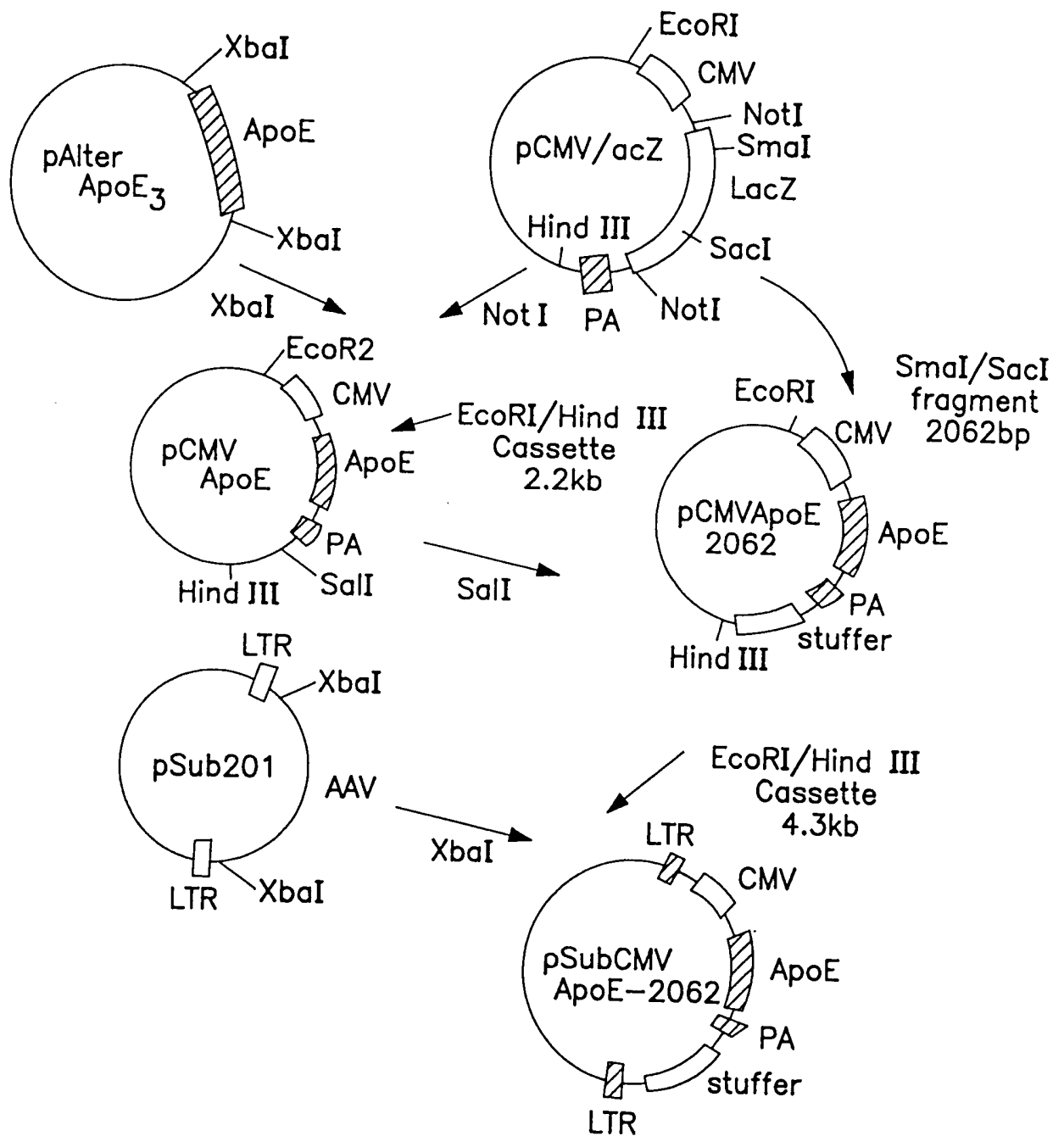


FIG. 13

# PCT

## REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference  
(if desired) (12 characters maximum) GNVPN.019CIP2PCT

**Box No. I TITLE OF INVENTION**

Method for Recombinant Adeno-Associated Virus-Directed Gene Therapy

**Box No. II APPLICANT**

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

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Suite 300  
Philadelphia, Pennsylvania 19104-3147  
United States of America

☐ This person is also inventor.

Telephone No.

215-573-4500

Facsimile No.

215-898-9519

Teleprinter No.

State (i.e. country) of nationality:

United States of America

State (i.e. country) of residence:

United States of America

This person is applicant for the purposes of:

☐

all designated States

☒

all designated States except the United States of America

☐

the United States of America only

☐

the States indicated in the Supplemental Box

**Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)**

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

Wilson, James M.  
1350 N. Avignon Drive  
Gladwyne, Pennsylvania 19035  
United States of America

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only (If this check-box is marked, do not fill in below.)

State (i.e. country) of nationality:

United States of America

State (i.e. country) of residence:

United States of America

This person is applicant for the purposes of:

☐

all designated States

☐

all designated States except the United States of America

☒

the United States of America only

☐

the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

**Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE**

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:

☒

agent

☐

common representative

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

Kodroff, Cathy A.  
Howson and Howson  
Spring House Corporate Center  
P.O. Box 457  
Spring House, Pennsylvania 19477  
United States of America

Telephone No.

215-540-9200

Facsimile No.

215-540-5818

Teleprinter No.

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

See Notes to the request form

If none of the following six boxes is used, this sheet is not to be included in the request.

Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)</i>  Fisher, Krishna J. 2338 Nashville Avenue New Orleans, Louisiana 70115 United States of America	This person is: <input type="checkbox"/> applicant only <input checked="" type="checkbox"/> applicant and inventor <input type="checkbox"/> inventor only <i>(If this check-box is marked, do not fill in below.)</i>
--	--

State (i.e. country) of nationality: United States of America	State (i.e. country) of residence: United States of America
--	--

This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input checked="" type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box
---

Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)</i>  	This person is: <input type="checkbox"/> applicant only <input type="checkbox"/> applicant and inventor <input type="checkbox"/> inventor only <i>(If this check-box is marked, do not fill in below.)</i>
---	---

State (i.e. country) of nationality:	State (i.e. country) of residence:
--------------------------------------	------------------------------------

This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box
--

Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)</i>  	This person is: <input type="checkbox"/> applicant only <input type="checkbox"/> applicant and inventor <input type="checkbox"/> inventor only <i>(If this check-box is marked, do not fill in below.)</i>
---	---

State (i.e. country) of nationality:	State (i.e. country) of residence:
--------------------------------------	------------------------------------

This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box
--

Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)</i>  	This person is: <input type="checkbox"/> applicant only <input type="checkbox"/> applicant and inventor <input type="checkbox"/> inventor only <i>(If this check-box is marked, do not fill in below.)</i>
---	---

State (i.e. country) of nationality:	State (i.e. country) of residence:
--------------------------------------	------------------------------------

This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box
--

<input type="checkbox"/> Further applicants and/or (further) inventors are indicated on another continuation sheet.
---

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) (mark the applicable check-boxes; at least one must be marked):

Regional Patent

- ☒ AP ARIPO Patent: GH Ghana, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☒ EA Eurasian Patent: AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ EP European Patent: AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☒ OA OAPI Patent: BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT (if other kind of protection or treatment desired, specify on dotted line)

National Patent (if other kind of protection or treatment desired, specify on dotted line):

- |  |  |
|--|--|
| <input checked="" type="checkbox"/> AL Albania                               | <input checked="" type="checkbox"/> LV Latvia                                    |
| <input checked="" type="checkbox"/> AM Armenia                               | <input checked="" type="checkbox"/> MD Republic of Moldova                       |
| <input checked="" type="checkbox"/> AT Austria                               | <input checked="" type="checkbox"/> MG Madagascar                                |
| <input checked="" type="checkbox"/> AU Australia                             | <input checked="" type="checkbox"/> MK The former Yugoslav Republic of Macedonia |
| <input checked="" type="checkbox"/> AZ Azerbaijan                            |  |
| <input checked="" type="checkbox"/> BA Bosnia and Herzegovina                | <input checked="" type="checkbox"/> MN Mongolia                                  |
| <input checked="" type="checkbox"/> BB Barbados                              | <input checked="" type="checkbox"/> MW Malawi                                    |
| <input checked="" type="checkbox"/> BG Bulgaria                              | <input checked="" type="checkbox"/> MX Mexico                                    |
| <input checked="" type="checkbox"/> BR Brazil                                | <input checked="" type="checkbox"/> NO Norway                                    |
| <input checked="" type="checkbox"/> BY Belarus                               | <input checked="" type="checkbox"/> NZ New Zealand                               |
| <input checked="" type="checkbox"/> CA Canada                                | <input checked="" type="checkbox"/> PL Poland                                    |
| <input checked="" type="checkbox"/> CH and LI Switzerland and Liechtenstein  | <input checked="" type="checkbox"/> PT Portugal                                  |
| <input checked="" type="checkbox"/> CN China                                 | <input checked="" type="checkbox"/> RO Romania                                   |
| <input checked="" type="checkbox"/> CU Cuba                                  | <input checked="" type="checkbox"/> RU Russian Federation                        |
| <input checked="" type="checkbox"/> CZ Czech Republic                        | <input checked="" type="checkbox"/> SD Sudan                                     |
| <input checked="" type="checkbox"/> DE Germany                               | <input checked="" type="checkbox"/> SE Sweden                                    |
| <input checked="" type="checkbox"/> DK Denmark                               | <input checked="" type="checkbox"/> SG Singapore                                 |
| <input checked="" type="checkbox"/> EE Estonia                               | <input checked="" type="checkbox"/> SI Slovenia                                  |
| <input checked="" type="checkbox"/> ES Spain                                 | <input checked="" type="checkbox"/> SK Slovakia                                  |
| <input checked="" type="checkbox"/> FI Finland                               | <input checked="" type="checkbox"/> SL Sierra Leone                              |
| <input checked="" type="checkbox"/> GB United Kingdom                        | <input checked="" type="checkbox"/> TJ Tajikistan                                |
| <input checked="" type="checkbox"/> GE Georgia                               | <input checked="" type="checkbox"/> TM Turkmenistan                              |
| <input checked="" type="checkbox"/> GH Ghana                                 | <input checked="" type="checkbox"/> TR Turkey                                    |
| <input checked="" type="checkbox"/> HU Hungary                               | <input checked="" type="checkbox"/> TT Trinidad and Tobago                       |
| <input checked="" type="checkbox"/> IL Israel                                | <input checked="" type="checkbox"/> UA Ukraine                                   |
| <input checked="" type="checkbox"/> IS Iceland                               | <input checked="" type="checkbox"/> UG Uganda                                    |
| <input checked="" type="checkbox"/> JP Japan                                 | <input checked="" type="checkbox"/> US United States of America                  |
| <input checked="" type="checkbox"/> KE Kenya                                 | <input checked="" type="checkbox"/> continuation-in-part                         |
| <input checked="" type="checkbox"/> KG Kyrgyzstan                            | <input checked="" type="checkbox"/> UZ Uzbekistan                                |
| <input checked="" type="checkbox"/> KP Democratic People's Republic of Korea | <input checked="" type="checkbox"/> VN Viet Nam                                  |
|  | <input checked="" type="checkbox"/> YU Yugoslavia                                |
| <input checked="" type="checkbox"/> KR Republic of Korea                     | <input checked="" type="checkbox"/> ZW Zimbabwe                                  |
| <input checked="" type="checkbox"/> KZ Kazakstan                             |  |
| <input checked="" type="checkbox"/> LC Saint Lucia                           |  |
| <input checked="" type="checkbox"/> LK Sri Lanka                             |  |
| <input checked="" type="checkbox"/> LR Liberia                               |  |
| <input checked="" type="checkbox"/> LS Lesotho                               |  |
| <input checked="" type="checkbox"/> LT Lithuania                             |  |
| <input checked="" type="checkbox"/> LU Luxembourg                            |  |

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

In addition to the designations made above, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except the designation(s) of \_\_\_\_\_.

The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. (Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)

**Supplemental Box. Use this box in the following cases:**

- (i) *if more than three persons are involved as applicants and/or inventors; in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III;*
- (ii) *if, in Box No. II or any of the sub-boxes of Box No. III, the indication "the States indicated in the 'Supplemental Box,'" is checked; in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State or States (or EP or OA, if applicable) for the purposes of which he/she/it is applicant;*
- (iii) *if, in Box No. II or any of the sub-boxes of Box No. III, a person indicated as "applicant and inventor" or "inventor only" is not inventor for the purposes of all designated States or for the purposes of the United States of America; in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor and, next to such name, the State or States (or EP or OA, if applicable) for the purposes of which the named person is inventor;*
- (iv) *if there is more than one agent and their addresses are not the same; in such case, write "Continuation of Box No. IV" and indicate for each additional agent the same type of information as required in Box No. IV;*
- (v) *if, in Box No. V, the name of any country (or OAPI) is accompanied by the indication "patent of addition," "certificate of addition," or "inventor's certificate of addition," or if, in Box No. V, the name of the United States of America is accompanied by an indication "Continuation" or "Continuation-in-part"; in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of parent title or filing of parent application;*
- (vi) *if there are more than three earlier applications whose priority is claimed; in such case, indicate "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;*
- (vii) *if, in any of the Boxes, the space is insufficient to furnish all the information; in such case, write "Continuation of Box No...." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient;*
- (viii) *if the applicant intends to claim, in respect of any designated Office, the benefit of provisions of the national law concerning non-prejudicial disclosures or exceptions to lack of novelty; in such case, write "Statement Concerning Non-prejudicial Disclosures or Exceptions to Lack of Novelty" and furnish that statement below.*

Continuation of Box IV

Kita, Stanley B.  
Smith, George A., Jr.  
Bak, Mary E.  
Bak, William

All above attorneys are members of the firm of Howson and Howson.  
Address of all is indicated in Box IV.

Continuation of Box V

United States of America - continuation-in-part of

USSN 08/708,188; filed 06 September 1996  
(06/09/96)

USSN 08/729,061; filed 10 October 1996  
(10/10/96)

**Box No. VI PRIORITY CLAIM (IF ANY).** The priority of the following earlier application(s) is/are claimed:

Country (country in which it was filed if national application; one of the countries for which it was filed if regional or international application)	Filing Date (day, month, year)	Application No.	Office of filing (fill in only if the earlier application is an international application or a regional application)
United States (1) of America	06 September 1996 (06/09/96)	08/708,188	
United States (2) of America	10 October 1996 (10/10/96)	08/729,061	
(3)			

(Letter codes may be used to indicate country and/or Office of filing)

When the earlier application was filed with the Office which, for the purposes of the present international application, is the receiving Office, the applicant may, *against payment of the required fee*, ask the following:

☒ the receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the above-mentioned earlier application/of the earlier applications identified above by the numbers (insert the applicable numbers) 1 & 2.

**Box No. VII EARLIER SEARCH (IF ANY).** Fill in where a search (international, international-type or other) by the International Searching Authority has already been requested (or completed) and the said Authority is now requested to base the international search, to the extent possible, on the results of the said earlier search. Identify such search or request either by reference to the relevant application (or the translation thereof) or by reference to the search request.

International application number or number and country (or regional Office) of other application:

International/regional/national filing date:

Date of request for search:

Number (if available) given to search request:

**Box No. VIII SIGNATURE OF APPLICANT(S) OR AGENT**

By:

Cathy A. Kodroff  
Cathy A. Kodroff  
Attorney for Applicants

If the present Request form is signed on behalf of any applicant by an agent, a separate power of attorney appointing the agent and signed by the applicant is required. If in such case it is desired to make use of a general power of attorney (deposited with the receiving Office), a copy thereof must be attached to this form.

**Box No. IX CHECK LIST (To be filled in by the Applicant)**

This international application contains the following number of sheets:

- |                |       |                  |
|----------------|-------|------------------|
| 1. request     | _____ | 5 sheets         |
| 2. description | _____ | 53 sheets        |
| 3. claims      | _____ | 2 sheets         |
| 4. abstract    | _____ | 1 sheets         |
| 5. drawings    | _____ | 13 sheets        |
| <b>Total</b>   |       | <b>74 sheets</b> |

Figure number \_\_\_\_\_ of the drawings (if any) is suggested to accompany the abstract for publication.

This international application as filed is accompanied by the items marked below:

- ☐ separate signed power of attorney
- ☐ copy of general power of attorney
- ☐ priority document(s) (see Box No. VI)
- ☐ receipt of the fees paid or revenue stamps
- ☒ cheque for the payment of fees
- ☒ request to charge deposit account
- ☒ other document (specify) sequence listing  
fee calculation sheet  
certification under 37 CFR 1.10

(The following is to be filled in by the receiving Office)

- Date of actual receipt of the purported international application:
- Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
- Date of timely receipt of the required corrections under Article 11 of the PCT:
- Drawings ☐ Received ☐ No Drawings

(The following is to be filled in by the International Bureau)

Date of receipt of the record copy:

## INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern

lication No

PCT/US 97/15692

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9534670 A	21-12-95	US 5658785 A AU 3124395 A CA 2192215 A EP 0764213 A	19-08-97 05-01-96 21-12-95 26-03-97
US 5436146 A	25-07-95	NONE	
WO 9726337 A	24-07-97	WO 9726336 A	24-07-97
WO 9712050 A	03-04-97	AU 7246796 A	17-04-97

## INTERNATIONAL SEARCH REPORT

Internat. Patent No.

PCT/US 97/15692

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/86 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 34670 A (CHILDRENS HOSPITAL INC) 21 December 1995 see page 5, line 16 - page 9, line 12 see especially page 9, first line see examples 4-7	1-10
X	COOVERT D D ET AL: "GENE THERAPY FOR MUSCLE DISEASES" CURRENT OPINION IN NEUROLOGY, vol. 7, no. 5, October 1994, pages 463-470, XP000673519 see the whole document	1-10
A	US 5 436 146 A (SHENK THOMAS E ET AL) 25 July 1995 see page 5, line 55 - page 6, line 13 see column 9, line 47 - line 62 -/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*G\* document member of the same patent family

Date of the actual completion of the international search

2 February 1998

Date of mailing of the international search report

04.03.98

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Sitch, W



## INTERNATIONAL SEARCH REPORT

Inter. Application No

PCT/US 97/15692

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SAMULSKI R J ET AL: "HELPER-FREE STOCKS OF RECOMBINANT ADENO-ASSOCIATED VIRUSES NORMAL INTEGRATION DOES NOT REQUIRE VIRAL GENE EXPRESSION" JOURNAL OF VIROLOGY, vol. 63, no. 9, 1 September 1989, pages 3822-3828, XP000283159 see page 3822 see abstract ---	
P,X	WO 97 26337 A (AVIGEN INC ;UNIV JOHNS HOPKINS (US)) 24 July 1997 see page 6, line 9 - page 9, line 5 see page 20, line 2 - page 34, line 5 ---	1-10
P,X	HERZOG ET AL: "STABLE GENE TRANSFER AND EXPRESSION OF HUMAN BLOOD COAGULATION FACTOR IX AFTER INTRAMUSCULAR INJECTION OF RECOMBINANT ADENO-ASSOCIATED VIRUS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES,USA, vol. 94, May 1997, pages 5804-5809, XP002054071 see the whole document ---	1-10
P,X	FISHER ET AL: "RECOMBINANT ADENO-ASSOCIATED VIRUS FOR MUSCLE DIRECTED GENE THERAPY" NATURE MEDICINE, vol. 3, no. 3, March 1997, pages 306-312, XP002054072 see the whole document ---	1-10
P,X	KESSLER ET AL: "GENE DELIVERY TO SKELETAL MUSCLE RESULTS IN SUSTAINED EXPRESSION AND SYSTEMIC DELIVERY OF A THERAPEUTIC PROTEIN" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES,USA, vol. 93, November 1996, pages 14082-14087, XP002054073 see the whole document ---	1-10
P,X	WO 97 12050 A (UNIV ROCKEFELLER ;KAPLITT MARTIN J (US); DIETRICH EDWARD B (US)) 3 April 1997 see page 5, line 29 - page 9, line 21 see page 26; example 1 -----	1-10

# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/US 97/15692

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

Remark : Although claims 7-10 partially, in so far as they relate to in vivo methods, are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.